ABSTRACT

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CHARACTERIZATION OF ADENOSINE TRANSPORT SITES IN GUINEA PIG CARDIAC MITOCHONDRIA

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The studies reported in this investigation involved the characterization of the nucleoside transport system at the subcellular level in cardiac tissue, in an attempt to augment the knowledge of adenosine's role in cardiovascular function. A protocol was developed that produced a substantially pure mitocondrial fraction. Kinetic and pharmacologic studies showed that [^{3}H]NBMPR binds to and labels populations of binding sites that appear to recognize adenosine as a probable substrate. Results from this data provide analytical probes for further binding site studies. Therefore, additional investigation is warranted in order to fully understand the interactions of adenosine and assess the mechanism by which the nucleoside produces its physiologic effects in the heart.

CHARACTERIZATION OF ADENOSINE TRANSPORT SITES IN GUINEA PIG CARDIAC MITOCHONDRIA

A DISSERTATION

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LIST OF ABBREVIATIONS

ADP	Adenosine 5' diphosphate
AMP	Adenosine 5' monophosphate
ATPase	Adensoine triphosphatase
APNEA	N^6-2-(4-Aminophenyl)ethyl)adenosine
AZPNEA	N^6-2-4-azidophenyl)ethyl)adenosine
B/F	Bound/Free
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CGS 21680	(2-(p-(carboxyethyl)phenethylamino)-5'-N-ethyl-carboxamidoadenosine
2-CADO	2-Chloroadenosine
CHA	N^6-Cyclohexyladenosine
CNS	Central Nervous System
CPA	N^6-Cyclopentyladenosine
CPCA	5'-(N-cyclopropyl)-carboxamidoadenosine
CV 1808	2-Phenylaminoadenosine
DPR	Dipyridamole
EGTA	Ethyleneglycol-bis tetraacetic acid
f/mol	Fentamole
g	Gram
IC_{50}	50% Inhibition Concentration
K_{+1}	Association rate constant
K_{-1}	Dissociation rate constant

K_D	Dissociation constant
K_{obs}	Initial rate constant
M	Molar concentration
min	Minute
mCi	Millicuri
MECA	5'-N-Methylcarboxamidoadenosine
mg	Milligram
ml	Milliliter
mol	Mole
NBMPR	Nitrobenzylthioinosine
NBTGR	Nitrobenzylguanosine
NECA	5'-N-Ethylcarboxamidoadenosine
nM	Nanomolar
NTS	Nucleoside Transport System
PAPA-APEC	(^{125}I-2-[4-(2-(2-[4-aminophenyl) methylcarbonylamino]ethylaminocarbonyl]-ethyl)phenyl]ethylamino-5'-N-ethylcarbox amidoadenosine
R-APHIA	R-2-azido-N^6-p-hydroxyphenylisopropyladeno sine
R-PIA	R-N^6-Phenylisopropyladenosine
S-PIA	S-N^6-Phenylisopropyladenosine
TCA	Trichloroacetic acid
TEM	Transmission electron microscope

$T_{\frac{1}{2}}$	Half time dissociation
Tris	Tris (hydroxymethyl)aminomethane
µM	Micromolar
µMol	Micromole
w/v	Weight/volume
x g	Relative centrifugal force

CHAPTER I

INTRODUCTION

Adenosine, a naturally occurring purine nucleoside metabolite, exerts dramatic physiological effects on the cardiovascular system. These effects have been reported to modulate processes such as vascular blood flow (Berne, 1980); platelet aggregation (Dawicki et al., 1985; Stiles, 1990); myocardial contractility (Belardinelli et al., 1983; 1989); neuromodulation, lymphocyte differentiation and lipolysis (Stiles, 1990).

It has been recognized that the nucleoside transport system (NTS), located in the plasma membrane, is a principal regulatory mechanism for the cardiovascular effects of adenosine. It is this transport mechanism that mediates the rapid uptake of adenosine into cells, thereby shortening its biological half-life and terminating the actions of the nucleoside (Plagemann and Wohlueter, 1980; Paterson et al., 1981). This rapid transport of adenosine across the plasma membranes occurs prior to its enzymatic inactivation. Thus, only trace amounts of the nucleoside is found in the extracellular fluids, even though it is continuously being produced.

The short-lived actions of adenosine, however, can be enhanced or potentiated by inhibiting its transport into cells (Stafford, 1966; Kolassa et al., 1971; Belardinelli et al., 1982). Consequently, a variety of compounds have been found to inhibit nucleoside transport which include agents such as the N^6-substituted 6-thiopurine nucleosides nitrobenzylthioinosine (NBMPR) and nitrobenzylthioguanosine (NBTGR) and other inhibitors such as dipyridamole (DPR), hexobendine, and dilazep (Olsson et al., 1972; Kolassa and Pfleger, 1975; Kolassa et al., 1978; Paterson et al., 1980; Lee and Jarvis, 1986).

Although information has been provided to explain how the actions of adenosine are potentiated, its role in cardiovascular function and the mechanism(s) of its actions to date have not been clearly defined. It is believed, however, that various physiological functions of adenosine in heart and other tissues are mediated by interactions with specific extracellular membrane receptors that are coupled to membrane-bound adenylate cyclase. These receptors are capable of inhibiting or stimulating the enzyme. Moreover, studies of adenosine antagonists such as aminophylline, various adenosine analogs and xanthine derivatives support evidence of these

cell surface receptors (Alfonso, 1970 and Olsson et al., 1977), which are located in coronary smooth muscle and atrial ventricular muscle (Dutta and Mustafa, 1979; 1987; Goodman and Synder, 1982). Based on pharmacological and biochemical studies, these receptors have been classified as A_1 and A_2 receptor subtypes according to their ability, in certain tissues, to inhibit (A_1) or stimulate (A_2) adenylate cyclase (Olsson et al., 1977; Schrader et al., 1977; Burnstock, 1980), and on the rank order of potencies with which adenosine agonists compete with a given radioligand for binding to the receptors. Additionally, a "P" site, a putative intracellular site located on the catalytic unit of adenylate cyclase, has been reported to directly inhibit its activity via ribose-modified adenosine analogs. The physiological significance of this site is unknown, although it has been shown to be pharmacologically distinct from the adenosine receptors (Haslam et al., 1978). A number of adenosine analogs exist, however, that contain a desired selectivity for the different receptor subtypes, depending on the pharmacological effects of interest. Furthermore, adenosine receptors modulate adenylate cyclase activity via G proteins, G_s and G_i for the A_2 and A_1 adenosine receptors, respectively. A_1 receptors have been recently

reported to be coupled to additional effector systems including guanylate cyclase, potassium channels, and phosphoinositol metabolism (Kurachi et al., 1986; Kurtz, 1987; Ramkumar et al., 1988).

Presently, a limited amount of information is available on the cardiovascular nucleoside transport system. Although the importance of the NTS to cardiovascular effects of adenosine has been recognized, its relevance to the adenosine-mediated regulation of cardiovascular function has not been sufficiently investigated. Consequently, an understanding of the mechanism(s) involved in adenosine's modulation of blood flow and in cardiovascular function still warrants attention. Therefore, the major objective of this investigation is to evaluate the role of nucleoside transport inhibition as a means to delineate the role of adenosine in cardiovascular function.

Moreover, it is important to consider the functional significance of the nucleoside transporters in the actions of adenosine. Since it is not known if the distribution of nucleoside transporters and adenosine receptors is similar, it is hoped that studies in subcellular fractions in the heart will provide information that will assist in answering this question.

The author has chosen to examine the role of nucleoside transport inhibition in cardiovascular function via the characterization of site-specific binding of [^{3}H]NBMPR to adenosine transport sites in pure guinea pig cardiac mitochondrial fractions. Mitochondria comprise approximately 25-35% of tissue protein in the heart (Page and McCallister, 1973; Palmer et al., 1977; Sordahl and Stewart, 1980). They are the major source of ATP in the normally functioning heart (Neely and Morgan, 1974) and contain most of the enzymes involved in energy transduction. More than 90% of cellular AMP, a direct precursor for adenosine, is located in the mitochondrial compartments (Bunger et al., 1983).

Coronary blood flow has been proposed to be coupled to tissue oxygen demand and regulated by the release of adenosine. Since the mitochondria are the sites of oxygen utilization and have been reported to produce adenosine (Bukoski et al., 1983), it seems feasible that these organelles may contain nucleoside transporters or perhaps employ a mechanism by which adenosine may be transported. It is anticipated through this investigation that it will be determined if there is a subcellular basis for the nucleoside transport mechanism in myocardial function and

if mitochondria play a role in nucleoside transport in the heart.

In order to address the current issues concerning the cardiovascular nucleoside transport system, the following specific research objectives were designated: (1) the isolation and purification of mitochondrial fractions from the guinea pig heart; (2) the identification and assessment of purity of the mitochondrial fractions via enzyme assays: Na^+-K^+-ATPase activity, Ouabain-sensitive-Na^+-K^+-ATPase activity, Ca^{++}-Stimulated-Mg^{++}-ATPase activity, and Cytochrome C Oxidase activity and also by electron microscopy; (3) the localization and characterization of nucleoside transporters of isolated myocardial cardiac mitochondria, by radioligand binding in terms of association and dissociation kinetics; (4) the determination of the dose-response relationship of nucleoside transport inhibitors and adenosine analogs for the binding site; and (5) the comparison of [^{3}H]NBMPR binding parameters to those in crude guinea pig ventricular membranes.

It should be noted that site-specific binding studies using radioligand probes at the subcellular level require a pure fraction. In the past, it has been difficult to isolate a mitochondrial fraction from the heart with

sufficient purity, due to the makeup of cardiac tissue. As a result, the isolation of a relatively pure mitochondrial fraction will be of major priority in this investigation.

Finally, it is anticipated that data provided by this study will enhance our understanding of adenosine's actions and the role of the nucleoside transport system in cardiovascular function.

CHAPTER II

REVIEW OF LITERATURE

Adenosine and Nucleoside Transport: A General Overview

The nucleoside transport system is a facilitated diffusion carrier system that accepts a variety of purine and pyrimidine nucleosides and plays an integral role in regulating the nucleoside adenosine. Nucleoside transport refers to the mediated bidirectional passage of nucleoside molecules across the plasma membrane of cells via nucleoside-specific transporter elements (Paterson et al., 1981). Subsequently, a rapid nucleoside uptake process results and involves the cellular accumulation of a nucleoside permeant and its metabolic products.

Nucleoside transport occurs via a number of facilitated diffusion processes, some of which can be blocked by compounds that bind to nucleoside transport sites and inhibit transport. Using the nucleoside adenosine and vasodilator agents such as dipyridamole and dilazep (Mustafa, 1979), and the benzodiazepine compound diazepam (Clanachan and Marshall, 1980), early studies were done to investigate the inhibition of nucleoside

transport. Later investigations studying adenosine accumulation in guinea pig cardiac muscle (Baker and Clanachan, 1982), focused attention on the difficulty of actually measuring adenosine transport rates because of the rapid rate of adenosine uptake. Accordingly, drug-induced inhibition of adenosine accumulation could not be used as an indicator of adenosine transport inhibition. To circumvent the problem of measuring the rate of transport, the binding of [^{3}H]NBMPR to guinea pig cardiac membranes was described and suggested as an alternate method of investigating the NTS in the heart (Williams and Clanachan, 1982).

Although a large variety of compounds have been found to inhibit nucleoside transport, dipyridamole and the purine analogs NBMPR and NBTGR have been the most useful probes of the nucleoside transport mechanism (Paterson et al., 1981). NBMPR, which is among the most potent and best studied of the group of nucleoside transport inhibitors, had been tritium labeled and was used as a radioligand to study the nucleoside transport system in the guinea pig heart (Williams and Clanachan, 1982). NBMPR was reported to bind saturably and reversibly to a single class of high affinity sites in these membranes which exhibited binding characteristics similar to those

observed for human erythrocytes (Hammond et al., 1981). Other investigators used the triated NBMPR to study nucleoside transport in various systems. The radioligand was shown to bind to an apparently homogeneous population of binding sites in rat and guinea pig brain preparations in a rapid and specific manner (Marangos et al., 1982). Similarly, reversible and high affinity NBMPR binding to specific sites on nucleoside transport elements of HeLa cells, presumably on the plasma membrane, was also reported (Hammond and Clanachan, 1985).

Unlike NBMPR, dipyridamole has been used extensively as a vasodilator and is well characterized in animals for its ability to potentiate the actions of adenosine (Morgan and Marangos, 1987). Its inhibition characteristics of adenosine accumulation were studied and compared to those of NBMPR in rat and guinea pig synaptosomes. Data from these studies revealed inhibition of adenosine accumulation to be distinctly biphasic and monophasic by NBMPR and dipyridamole, respectively, in all preparations studied. Further investigations revealed that [^{3}H]-dipyridamole labeled substantially more binding sites per unit protein than [^{3}H]NBMPR in guinea pig cortical membranes as compared to rat tissue (Marangos et al., 1985; Deckert, 1985). These observations support the

hypothesis that both compounds can bind to functionally relevant sites and that different populations of nucleoside transporters exist in mammalian brain. Nucleoside transport systems differ among species and in tissues in a number of criteria (Parkinson and Clanachan, 1989). Sensitivity to inhibitors of nucleoside transport, primarily NBMPR and dipyridamole has been the basis by which facilitated diffusion systems have usually been subclassified. Hopkins and Goldie (1971) showed that dipyridamole does not potentiate adenosine effects in rat tissue. Additional studies revealed that dipyridamole, lidoflazine and hexobendine have significantly lower affinity for nucleoside transport systems in rat tissue than in other species, particularly guinea pig. Moreover, Williams and colleages (1984), compared the characteristics of the site-specific binding of [^{3}H]NBMPR to cardiac membranes of several mammalian species. The affinity of the radioligand for these sites and the maximal binding capacity of cardiac membranes for [^{3}H]NBMPR were both species dependent. Other investigations showed dipyridamole and dilazep to inhibit NBMPR binding in some Central Nervous System (CNS) preparations in a biphasic manner (Hammond and Clanachan, 1984; 1985). These membrane preparations possessed

apparent uniform populations of NBMPR binding sites.

Recently, regional subtypes of facilitated diffusion nucleoside transport systems have been discovered and identified. Systems of high and low sensitivity of NBMPR were identified in several lines of cultured cells (Paterson et al., 1987) and to dipyridamole in guinea pig and rat myocytes, respectively. More recently, Shank and Baldy (1990) reported differences in adenosine transport systems of the rat and guinea pig. NBMPR-sensitive systems were pharmacologically different in the two species in that certain compounds (i.e., dipyridamole and mioflazine) proved to be more potent inhibitors in the guinea pig.

Investigations of cardiovascular adenosine transport systems are steadily being conducted. [^{3}H]NBMPR binding studies were done in smooth muscle and endothelial cells (Williams et al., 1990). The presence of nucleoside transport elements in cultured primate vascular smooth muscle, bovine aortic endothelial, and human umbilical vein endothelial cells were reported with multiple [^{3}H]NBMPR binding sites being detected in human umbilical vein endothelium. Based upon their findings, the investigators concluded that the actions of adenosine in

these tissues are enhanced by dipyridamole and other agents and that these effects can be attributed, in part, to interaction with the nucleoside transporter elements in vascular smooth muscle and endothelium.

Physiological Effects of Adenosine

The profound physiological effects of adenosine was first recognized by Drury and Szent-Gyorgyi approximately 60 years ago when it was demonstrated that the nucleoside linked cardiac metabolism to coronary and vascular smooth muscle tone. Adenosine's effects are not limited, however, to the cardiovascular system. Almost all organs of the body are regulated to some extent by its release (Table 1). Consequently, the diverse roles of adenosine and its physiological properties, along with the potential therapeutic value of adenosine transport inhibitors, stimulated the current interest in adenosine transport systems.

The most prominent physiologic role of adenosine has been in cardiovascular function where its effects cause vasodilation, hypotension, and cardiac depression (Kusachi et al., 1983; Leung et al., 1983).

From a pathophysiological perspective, adenosine has recently been reported to be released as a pharmacologic

Table 1. Physiological Functions of Adenosine

System	Response/Effects of Adenosine	Reference
Adrenals	Stimulate steroidogenesis	Londos et al., 1980
Adipocytes	Inhibit lipolysis; stimulate glucose oxidation	Souness and Sanchez, 1981; Stiles, 1986; Ramkumar et al., 1988
Brain	Mediates sedation; Regulates blood flow	Synder et al., 1981; Winn et al., 1981
Endothelial Cells	Stimulate proliferation and migration of endothelial cells (angiogenesis)	Meininger et al., 1981
Heart	Regulates cardiac rate and vascular muscle tone	Drury and Szent-Gyorgyi, 1929
	Regulates the force of contractility and electrophysiologic properties of the heart	Belhassen and Pelleg, 1984; Belardinelli et al., 1989
	Pharmacologic agent for treatment of cardiac dysrhythmias	Stiles, 1990
	Regulates coronary blood flow and induces relaxation	Drury and Szent-Gyorgyi, 1929; Berne, 1908; Osswald, 1988

"Table 1 (Continued)."

System	Response/Effects of Adenosine	Reference
Kidney	Causes vasoconstriction	Spielman and Thompson, 1982
	Regulates renin release and vascular tone	Berne, 1980; Stiles, 1986
Lymphocytes	Modulate lymphocyte differentiation	Dawicki et al., 1985
Neurons	Suppress neurotransmitter release	Verhage et al., 1977
	Inhibit spontaneous activity of central neurons	Phillips et al., 1979
Platelets	Modulate/inhibit platelet aggregation	Born, 1964; Verhage et al., 1977; Cusack and Hourand, 1981
Skeletal and Smooth Muscle	Induce relaxation	McKenzie et al., 1977; Buckle and Spence, 1981
Vasculature	Causes vasodilation; alters cAMP and adenylate cyclase activity; acts as an immunosuppressant	Rubio et al. 1969; Stiles, 1986
Lungs	Induce bronchoconstriction and potentiate asthma	Fredholm, 1980; Ukena et al., 1985

agent for the treatment of cardiac dysrhythmias (Stiles, 1990) in addition to functioning as a hypotensive agent in hypertension.

Additionally, the antilipolytic, antithrombotic, and antispasmodic actions of adenosine result in varied responses that are linked to adenylate cyclase activity in different cell types. The inhibition of adenylate cyclase in adipocytes activates the antilipolytic actions; the activation of adenylate cyclase in platelets by adenosine results in the inhibition of platelet aggregation; and the activation of muscle adenylate cyclase produces depressant effects that are linked to the antispasmodic and vasodilatory actions of adenosine. Secondly, adensoine has been demonstrated to function as an endogenous neuromodulator in the mammalian CNS (Dunwiddie, 1985) and to exert inhibitory effects on neurotransmission and spontaneous activity of the CNS neurons (Daly, 1982). The mechanism for the inhibitory actions was thought to involve calcium ions and the presynaptic inhibition of adenylate cyclase. The inhibitory effects of adenosine on cholinergic and noradrenergic transmitter release were demonstrated to be reversed by calcium ions. As a result, adenosine and its analogs came to be referred to as calcium antagonists. A third effect of adenosine is its

stimulatory action in adrenal cells that lead to steroidogenesis, again as a result of adenylate cyclase activity. More recently, studies of adenosine's vasodilatory effects were extended to endothelial tissue. It was observed that adenosine induced stimulation of adenylate cyclase activity in endothelial cells via its direct actions on the enzymes's catalytic P site subunit (Legrand et al., 1990). Further studies revealed that adenosine also stimulated the proliferation and migration of bovine aortic or coronary microvascular endothelial cells (in vitro) in response to hypoxia (Meininger et al., 1988; 1990).

Finally, the actions of adenosine can also produce vasconstrictory effects. In the lungs, the actions of adenosine involves bronchoconstriction. It has been concluded that adenosine exerts its actions on airways by modulating mast cell degranulation (Cushley and Holgate, 1985) and by inducing immediate bronchoconstriction in asthmatic airways (Fredholm, 1980; Ukenar et al., 1985). Further studies by Holgate and colleages to determine the nature of bronchoconstriction produced findings that suggested that adenosine acts at a cell surface receptor, probably of the A_2 type, to elicit bronchoconstriction responses. Another organ in which the effects of

adenosine result in vasoconstriction instead of vasodilation is the kidney. Unlike the lungs, however, the mechanisms involved in these vasoconstrictor actions are still poorly understood and require more investigation.

Adenosine Formation and Release

In contrast to the progress made in assessing the mechanisms of adenosine's actions, considerable controversy still exist concerning the sites of adenosine production and the mechanism of its release in the heart. Similarly, the significance of the metabolic pathways involved in the formation and inactivation of adenosine is still unresolved.

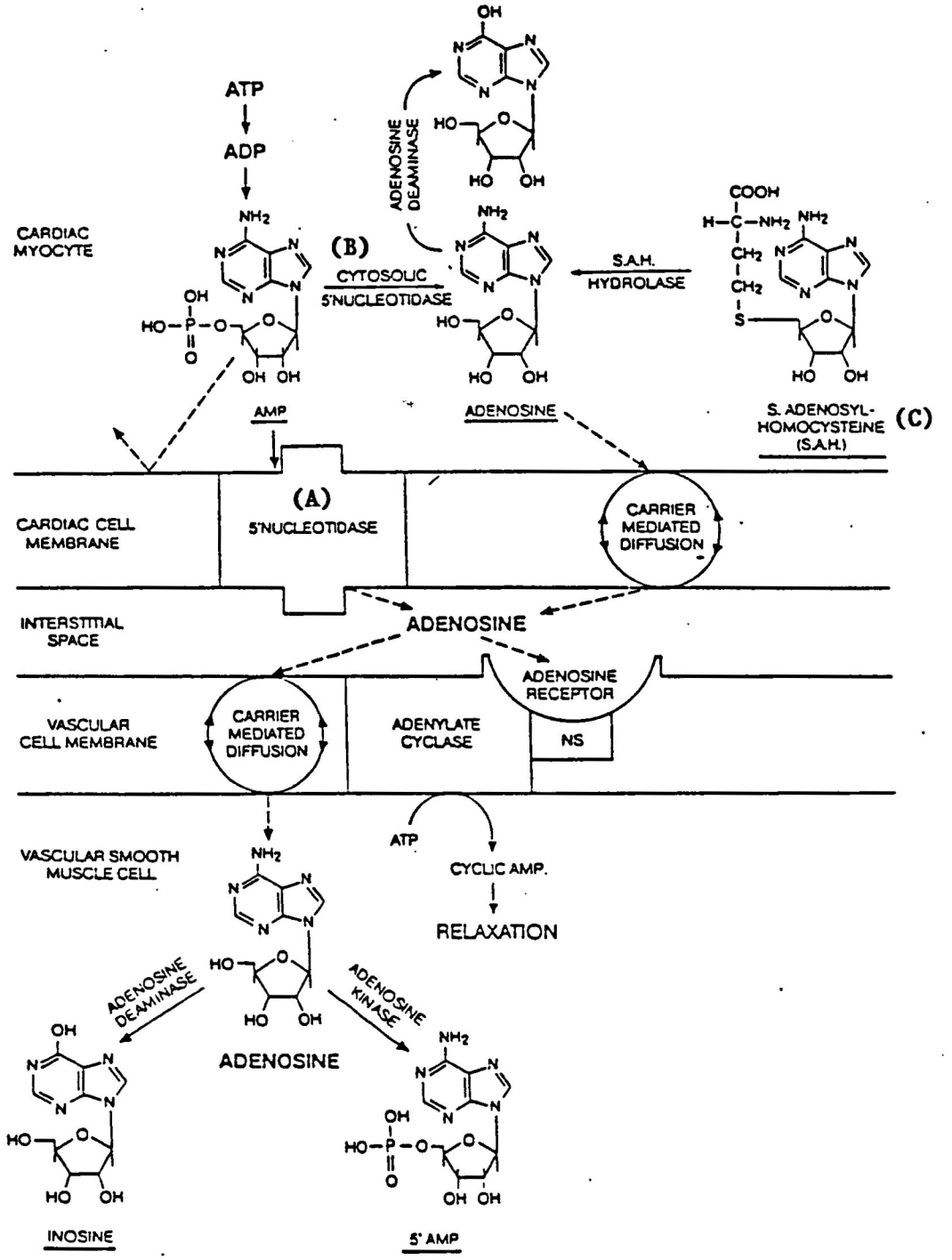
Three pathways (Fig. 1) have been proposed as potential sources of adenosine in the heart: (1) ecto-5'-nucleotidase (Frick and Lowenstein, 1976; Lowenstein et al., 1983); (2) cytosolic 5'-nucleotidase (Gibson and Drummond, 1972; Lowenstein et al., 1983; Itoh and Oka, 1985; Meghji et al., 1988) and (3) S-adenosylhomocysteine (Schrader et al., 1981). ATP utilization leads to increased cytosolic levels of 5'-nucleotidase. The cytosolic form of 5'-nucleotidase is currently thought to be more important in producing intracellular adenosine

Figure 1. **Schematic Diagram Showing the Pathways of Adenosine Formation, Production and Its Fate in the Heart (Adapted from Feigh, 1983).**

A) The ectoenzyme, 5-nucleotidase , dephosphorylates 5'-AMP to adenosine

B) Cytosolic 5'-nucleotidase produces an intracellular source of adenosine

C) S-adenylhomocysteine (SAH) produces adenosine via the transmethylation pathway.


ATP
ADP
CARDIAC MYOCYTE
(B)
CYTOSOLIC 5'NUCLEOTIDASE
ADENOSINE DEAMINASE
S.A.H. HYDROLASE
AMP
ADENOSINE
S. ADENOSYL-HOMOCYSTEINE (S.A.H.)
(C)
(A)
5'NUCLEOTIDASE
CARDIAC CELL MEMBRANE
CARRIER MEDIATED DIFFUSION
INTERSTITIAL SPACE
ADENOSINE
ADENOSINE RECEPTOR
VASCULAR CELL MEMBRANE
CARRIER MEDIATED DIFFUSION
ADENYLATE CYCLASE
NS
ATP
CYCLIC AMP.
RELAXATION
VASCULAR SMOOTH MUSCLE CELL
ADENOSINE DEAMINASE
ADENOSINE KINASE
ADENOSINE
INOSINE
5' AMP

than the membrane-bound ecto-enzyme (Van den Berghe et al., 1977; Itoh, 1981; Worku and Newby, 1983; Newby et al., 1987), even though its regulation remains to be fully explored. Alternatively, S-adenosylhomocysteine is hydrolyzed to adenosine. Adenosine is thought to leave the cardiac myocyte via carrier-mediated diffusion and enters the interstitial space where it is transported into a variety of cell types. Interactions on vascular smooth muscle is thought to increase cAMP levels and subsequent relaxation. Adenosine that is transported into cells is either deaminated or phosphorylated.

Regulation of Adenosine Receptors (Signal Transduction Mechanism)

The effects of drugs and hormones can be modulated by cells via cell surface receptors. Chronic exposure of these receptors to agonists can result in an uncoupling of the receptor from the effector system. This uncoupling may lead to the densensitization of the inhibitory effects of agonists or to an enhanced response to stimulatory agents (Rall and Wutherland, 1990; Williams, 1990).

Regulation of adenosine receptors has been linked to the signal transduction mechanism. It has been previously stated that adenosine can both stimulate and inhibit

adenylate cyclase activity via receptors that are pharmacologically distinct. The potency series of R-PIA>adenosine>NECA is distinctive for the A_1 (inhibitory) receptor whereas the reverse is true for the A_2 (stimulatory) receptor (Wolff et al., 1981). Rodbell (1980) reported that GTP (G) regulatory proteins were required by these receptors to mediate the effects of adenosine on adenylate cyclase activity. The involvement of the G-regulatory proteins in this process was confirmed by the use of the cholera and pertussis toxins which selectively modify ADP-ribosylation reactions. Cholera toxin labels the alpha-subunit of the G_s (stimulatory) subunit and the pertussis toxin labels and modifies the G_i (inhibitory) alpha-subunit. Cholera toxin enhances the stimulatory actions of the A_2 receptor (Lad et al., 1980) which causes prolonged activation of adenylate cyclase, whereas pertussis toxin eliminates the G_i-mediated activities which prevents the A_1 receptor from inhibiting adenylate cyclase (Hazeki and Ui, 1981). Thus the G proteins are responsible for inducing the continued stimulation of adenylate cyclase activity.

Recent evidence indicates that adenosine receptors are linked to signal transduction mechanisms other than the adenylate cyclase signaling system. These mechanisms

include effects of adenosine on: (1) plasma membrane ion channels (Changeux et al., 1984), (2) cardiac potassium channels (Kurachi et al., 1876), and (3) calcium channels (Peterson and Maruyana, 1984; Kameyama et al., 1985).

Ligands Used to Characterize Adenosine Receptors

A number of agonists and antagonists radioligands of diverse structures (Table 2) has been developed that bind to adenosine receptors (Research Biochemicals Incorp., 1990). These ligands have made possible the characterization of transport sites and receptors. Since 1980, several A_1 receptor selective radioligands have been developed but only a few selective A_2 receptor agonists have been reported. CV-1808 (2-phenylaminoadenosine), a coronary vasodilator from Takeda, was the first A_2 selective compound to be identified. CGS 21680 and its analog PAPA-APEC are the most selective A_2 high affinity agonists reported today (Barrington et al., 1989; Stiles, 1989) and are used as specific radioligand photoaffinity probes. Yeung and Green (1983) performed successful studies on the A_2 selective receptor using the nonspecific ligand, [^{3}H]-N-ethylcarbosyamido-adenosine (NECA). Likewise, A_1 selective photoaffinity ligands ^{125}I-APNEA and ^{125}I-AZPNEA were developed in 1985 (Stiles

Table 2. Adenosine Agonist and Antagonist Ligands Employed in the Characterization of Adenosine Receptors

Agonists	Selectivity
Adenosine	—
Adenosine amine congener (ADAC)	A_1
N^6-Benzyladenosine	$A_1 > A_2$
2-Chloroadenosine	$A_1 > A_2$
CV-1808	$A_2 > A_1$
N^6-Cyclohexyladenosine (CHA)	A_1
N^6-Cyclopentyladenosine (CPA)	A_1
5'-(N-Cyclopropyl)-carboxamidoadenosine	A_2
1-Deaza-2-chloro-N^6-cyclopentyladenosine	A_1
DPMA (PD-125944)	A_2
ENBA, (S)- (PD-126280)	A_1
5'-N-Ethylcarboxamidoadenosine (NECA)	$A_2 = A_1$
N^6-Methyladenosine	—
5'-N-Methylcarboxamidoadenosine	$A_2 > A_1$
1-Methylisoguanosine	A_1
2-Methylthio-ATP	$P2_x$
N^6-Phenyladenosine	$A_1 > A_2$
N^6-Phenylethyladenosine	$A_1 > A_2$
PIA, R(–)-	A_1
PIA, S(+)-	A_1
Antagonists	
Aminophylline	$A_2 = A_1$
7-(β-Chloroethyl)theophylline	—
8-Cyclopentyl-1,3-dimethylxanthine	A_1
8-Cyclopentyl-1,3-dipropylxanthine	A_1
1,3-Diethyl-8-phenylxanthine	A_1
1,3-Dimethylxanthine (Theophylline)	$A_1 > A_2$
1,7-Dimethylxanthine (Paraxanthine)	$A_1 > A_2$
3,7-Dimethylxanthine (Theobromine)	$A_1 > A_2$
1,3-Dipropyl-7-methylxanthine	$A_2 > A_1$
1,3-Dipropyl-8-*p*-sulfophenylxanthine	$A_1 > A_2$
7-(β-Hydroxyethyl)theophylline	—
3-Isobutyl-1-methylxanthine (IBMX)	$A_1 > A_2$
PACPX	A_1
8-Phenyltheophylline	A_1
3-(*n*-Propyl)-xanthine (Enprofylline)	$A_1 > A_2$
8-(*p*-Sulfophenyl)-theophylline	$A_1 > A_2$
1,3,7-Trimethylxanthine (Caffeine)	—
Xanthine amine congener (XAC)	A_1
Other	
AHPIA, R(–)-	A_1 photoaffinity
Dipyridamole	Uptake inhibitor

et al.) to probe the A_1 receptor and demonstrated binding that was reversible, saturable, stereospecific, and selective to this receptor in a variety of tissues. The A_1 receptor ligands used most include CHA, 2-CADO, and R-PIA, although the N-6 cyclopentyl analog of adenosine (CPA) is approximately 6-fold more selective that CHA as an A_1 ligand (Bruns, 1984; Daly, 1984). A second group of compounds, the xanthine-adenosine antagonists were developed and used to label A_2 sites and other "antagonist" recognition sites in brain tissue. The best known antagonists of adenosine receptors are theophylline, caffeine and theobromine.

Future Trends in Adenosine Research

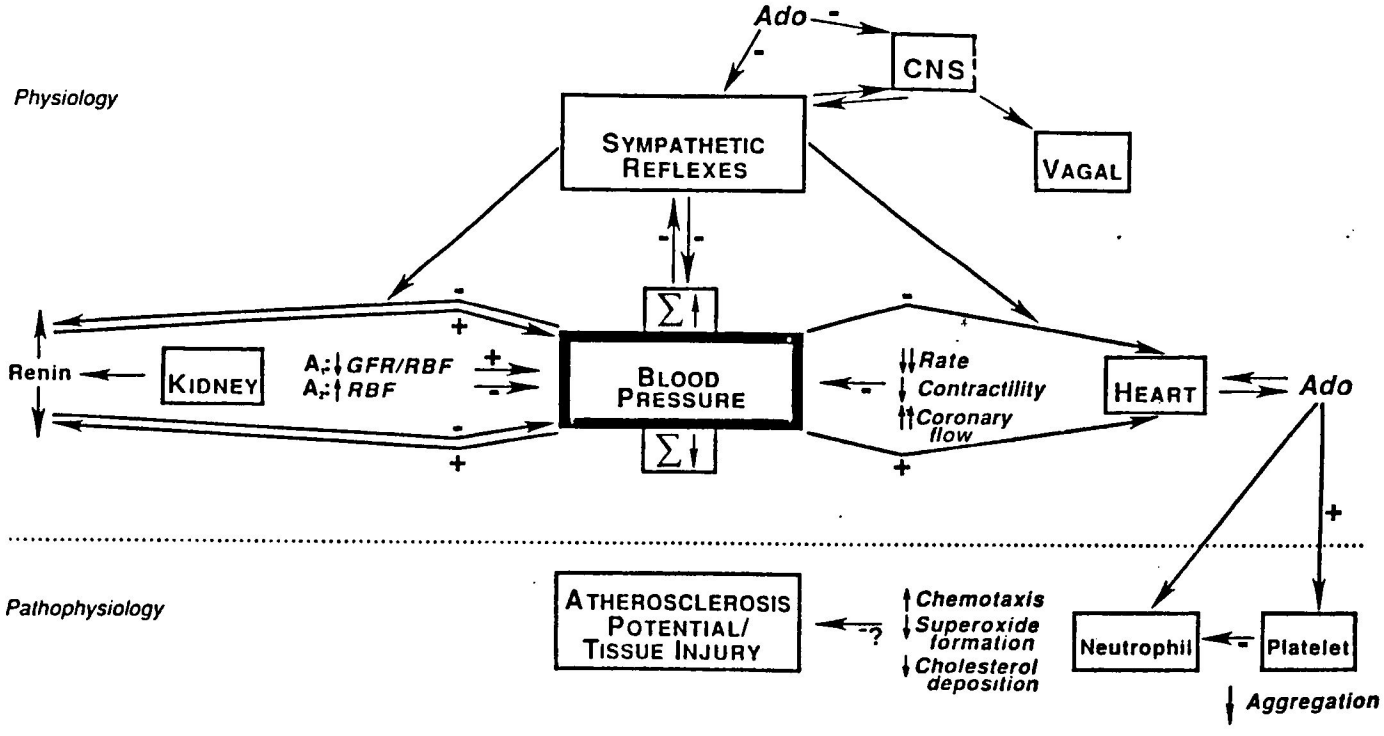
Since the actions of adenosine in regulating coronary blood flow was recognized by Drury and Szent-Gyorgi in 1929, a vast accumulation of information on the effects of adenosine in cardiovascular function has proven enlightening, but has failed to resolve the question of the nucleoside's role as a therapeutic agent or physiological modulator in cardiovascular function. To date, the goal to clearly define the nature of the nucleoside transport sites labeled by [^{3}H]NBMPR in the

heart has yet to be accomplished. However, receptor binding studies, the development of new ligands and the isolation and cloning of the two adenosine receptor subtypes (Stiles et al., 1985; Barrington et al., 1989) have provided data that can be instrumental in augmenting further investigations concerning the potential actions of adenosine. Likewise, the relationship of adenosine receptors to membrane G proteins, ion channels, and second-messenger systems (Cooper and Caldwell, 1989) has proven very valuable in investigating newer areas of adenosine research.

An abundance of data reveal adenosine to exert potent effects both directly and indirectly on cardiovascular function. Direct effects occur through the activation of A_1 and A_2 receptors in the heart and by receptor interactions in the peripheral vasculature, CNS and the kidney while indirect effects occur via its antiamine effects and its effects on renin production. Nevertheless, the complexities of these responses and their interrelationships still require further investigation. A schematic diagram of the potential effects of adenosine mediated responses is given in Fig. 2.

Figure 2. Schematic Diagram of the Potential Effects of Adenosine Mediated Responses.

The effects of adenosine on blood pressure are complex, occuring via both direct and indirect mechanisms. Ado = Adenosine. Vertical arrows indicate increases (↑) or decreases (↓) in the parameters indicated.


Physiology
Ado
CNS
SYMPATHETIC REFLEXES
VAGAL
Renin
KIDNEY
A_a↓ GFR/RBF
A_e↑ RBF
BLOOD PRESSURE
↓↓Rate
↓ Contractility
↑↑Coronary flow
HEART
Ado
Pathophysiology
ATHEROSCLEROSIS POTENTIAL/ TISSUE INJURY
↑Chemotaxis
↓Superoxide formation
↓Cholesterol deposition
Neutrophil
Platelet
↓ Aggregation

Adenosine's role as a hypotensive agent makes it an important research area of cardiovascular function. However, an understanding of its mechanisms of action and its interrelationships remains unclear. It is important that adenosine's pharmacological properties and pathophysiological role be thoroughly and aggressively investigated if a cohesive understanding of its cardiovascular parameters is to materialize.

CHAPTER III

MATERIALS AND METHODS

Animals

Male Heartley albino guinea pigs (400-450 g) were purchased from Buckberg Laboratory Animals, Inc. (Thomkins Cove, NY) and housed in the animal care facility of Morehouse School of Medicine.

Drugs and Chemicals

Drugs and radioligand for these studies were obtained from the following sources: [^{3}H]NBMPR (sp. act. 0.5 mCi/mole) from Dupont, New England Nuclear Company (Boston, MA); ferrocytochrome C, potassium ferricyanide, cytochrome c oxidase, adenosine, NECA, CHA, GTP, 2-Chloroadenosine, cAMP, cGMP, ATP, GDP, R-PIA, S-PIA, dipyridamole, CPA, NBTGR, EGTA, Ouabain, cGMP and papaverine, from Sigma Chemical Company (St. Louis, MO); theophylline, theobromine, caffeine, CV-1808, MECA, CPCA from Research Biochemical (Natick, MA); and Ecosint from National Diagnostic Inc. (Manville, NJ). All other materials were of the purest available grades and were

purchased from Fisher Scientific Company (Norcross, GA) and Electron Microscopy Science (Ft. Washington, PA).

Experimental Procedures

Isolation and Preparation of Cardiac Mitochondria

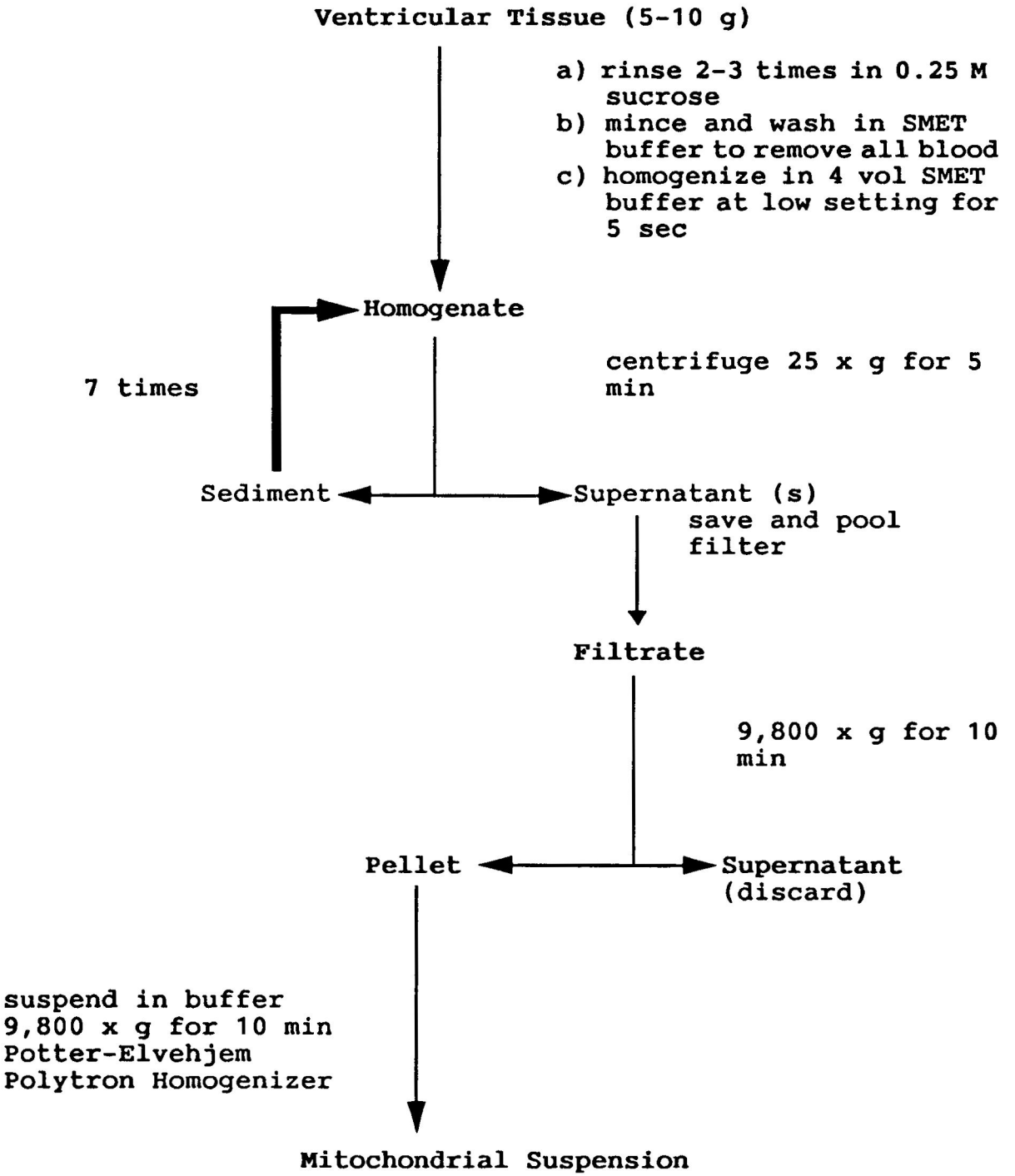
Mitochondria were isolated following a modified procedure of Jaqua-Stewart et al. (1979) and Chance and Hagihara (1963) as modified by Tyler and Gonze (1967). Male Hartley albino guinea pigs (400-450 g) were anesthetized with ethyl ether and decapitated. Hearts (5-10 g) were quickly excised, trimmed, and rinsed 2-3 times in 0.25 M sucrose. Ventricles were finely minced and washed in ice-cold SMET buffer that contained 0.25 M sucrose, 70 mM mannitol, 0.2 mM EGTA, and 5 mM Tris-HCl (pH 7.4) until all excess blood was removed. The minced tissue was homogenized briefly (5 sec) in 4 vol (w/v) of SMET buffer using a Polytron, Ultra Turrax Tissue Processor, at a rheostat setting of 4. The homogenate was centrifuged at 25 x g for 5 min. The supernatant was decanted and saved. The sediment was then subjected to a series of homogenizations and slow centrifugations as described above. All supernatants were saved and pooled, filtered and centrifuged at 9800 x g for 10 min. The mitochondrial pellets were washed twice by centrifugation at 9800 x g for 10 min each. The final pellets were

resuspended in 0.8 ml to 1 ml of SMET buffer per gram original tissue weight and homogenized in a motor-driven Potter-Elvehjem homogenizer to yield a uniform suspension. This suspension was subjected to homogenization with the Polytron, Ultra Turrax Tissue Processor, at a rheostat setting of 1 and then diluted to a protein concentration of 0.1-0.2 mg/ml and kept on ice for immediate use. All isolation procedures were carried out at 0-4^{o}C using freshly prepared mitochondrial fractions. Protein was assayed by the method of Lowry et al (1951). The entire isolation procedure is schematically described in Fig. 3.

Purification of Cardiac Mitochondria

The purity of the mitochondrial fraction was determined using enzyme markers and by electron microscopic examination. Cytochrome c oxidase was used as the marker enzyme to identify the fraction as being of mitochondrial origin, while Na^{+}-K^{+}-ATPase activity and Ouabain sensitive Na^{+}-K^{+}-ATPase activity (markers for the sarcolemma) and Ca^{++} sensitive-Mg^{++}-ATPase activity (marker for the sarcoplasmic reticulum) were used to assess membrane cross-contamination. The enzyme activities were compared in the total crude homogenate, pooled supernatant, and purified mitochondrial membranes. All assays were carried out at 0-4^{o}C except where

Figure 3. Isolation Scheme of Mitochondria from Guinea Pig Heart.


Ventricular Tissue (5-10 g)
a) rinse 2-3 times in 0.25 M sucrose
b) mince and wash in SMET buffer to remove all blood
c) homogenize in 4 vol SMET buffer at low setting for 5 sec
Homogenate
7 times
centrifuge 25 x g for 5 min
Sediment
Supernatant (s)
save and pool
filter
Filtrate
9,800 x g for 10 min
Pellet
Supernatant (discard)
suspend in buffer
9,800 x g for 10 min
Potter-Elvehjem
Polytron Homogenizer
Mitochondrial Suspension

indicated. The procedures used to assess the purity of the mitochondrial fractions are described below.

Marker Enzyme Assays

Cytochrome C Oxidase Assay

The activity of this enzyme was assayed by the spectrophotometric procedure of Wharton and Tzagoloff (1967). This assay measures the oxidation rate of ferrocytochrome c by following the decrease in absorbance of its alpha-band at 550 nm. A solution containing 0.10 ml of potassium phosphate buffer (0.01 M, pH 7.0), 0.07 ml 1% ferrocytochrome C (reduced cytochrome c), and 0.83 ml water was added to two cuvettes with a 10 mm light path. A blank cuvette was oxidized with 0.01 ml potassium ferricyanide. The reaction was initiated at 38°C by the addition of 10 µl tissue suspension to two cuvettes and 10 µl cytochrome c oxidase to two different cuvettes (protein concentration of 0.30 mg/ml). The decrease in absorbance was measured at 550 nm every 15 sec. Cytochrome c activity was defined in terms of the 1st-order velocity constant. The specific activity was calculated from the known concentration of cytochrome c and cytochrome oxidase in the assay mixture and the estimated first-order velocity constant (k).

Assay of ATPases

All assays were performed in triplicate at 37°C. ATPase activity was determined by the following procedure. Tissue samples (100 µg-200 µg) were incubated for 5 min in 1 ml final volume of a basic incubation medium. The medium for Mg^{++}-Na^{+}-K^{+}-stimulated and Ouabain sensitive ATPase activity contained 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, and 1 mM EGTA. Ca^{++}-stimulated-Mg^{++} dependent APTase incubation medium contained 2 mM $CaCl_2$ instead of EGTA. Total ATPase activity was measured by incubating tissue samples in a total activity buffer containing 10 mM KCl, 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 1mM EGTA, 3mM $CaCl_2$ and 0.30 mM Ouabain. The reaction was initiated after the 5 min incubation by the addition of 4 mM ATP and terminated after 10 min by the addition of 1 ml of 12% (w/v) tricholoroacetic (TCA), followed by the removal of the denatured protein via centrifugation at 2000 x g for 10 min. A 1 ml aliquot of the supernatant was assayed for inorganic phosphate using a colorimetric Fiske SubbaRow inorganic phosphate kit. The assay was calibrated by constructing a standard curve of known inorganic phosphorus concentration vs absorbance at 660 nm.

Electron Microscopy

Mitochondrial suspensions were fixed in 2.5% gluteraldehyde in 0.10 M cacodylate buffer, pH 7.4 for 24 h at 4°C. The fixed suspension was pelleted at 1,000 x g for 10 min. Pellets were washed twice in cacodylate buffer in 10% sucrose and then post-fixed in 2% osmium tetraoxide (O_sO_4) for 1 h. Dehydration was done in an ethanol series at room temperature with an interchange of propylene oxide followed by embedding in Epon 812. Thin sections were cut with a Porter-Blum ultramicrotome using glass knives and were doubled stained with uranyl acetate and lead citrate. The sections were examined with a Jeol JEM-1200 EX electron microscope.

Characterization of Mitochondrial Fractions

[^{3}H]NBMPR Binding to Intact Mitochondria

Binding assays were carried out in a final incubation volume of 1 ml that contained 0.50-0.55 ml Tris-HCl (pH, 7.4), 0.40 ml mitochondrial suspension in the absence or presence of 0.05 ml of a nonspecific displacer or NBMPR inhibitor and 0.05 ml of graded concentrations of [^{3}H]NBMPR. Binding was terminated after 1 h incubation (room temperature), by rapid vacuum filtration over Whatman GF/B filters with two 5 ml washes of ice-cold

Tris-HCl buffer (50 mM, pH 7.4). [^{3}H] Radioactivity associated with the fractions was measured by liquid scintillilation after filters were transferred into vials containing 5 ml of scintillation fluid (Ecosint) and counted in a Beckman LS5801 scintillation counter. Specific (saturable) [^{3}H]NBMPR binding was determined by subtracting nonspecific binding values (binding in the presence of the nonspecific displacer) from total [^{3}H]NBMPR binding (binding in the absence of the nonspecific displacer) values obtained from assays performed in duplicate.

Statistical Analysis

Saturation isotherms were analyzed by the least squares analysis of Scatchard (1949) plots and by the nonlinear, multipurpose curve fitting computer program LIGAND (Munson and Rodbard, 1980) to determine dissociation constants (K_D) and maximal number of binding sites (B_{max}) from the saturation data. K_D is expressed in nM and B_{max} as fmol/mg protein. IC_{50} values, which represent the concentration of the inhibitor/displacer that inhibits the specific displaceable binding of [^{3}]HNBMPR by 50%, were calculated from semilog plots employing 10 concentrations of the test compounds. These values were determined by the GraphPad InPlot computer program (GraphPad Intuitive Software for Science, San

Diego, CA). In addition, Hill coefficients (^{n}H) were determined from the slopes of Hill plots of saturation isotherms or competition data of [^{3}H]NBMPR according to the equation: log (B/B_{max}-B) = n log [L]-log K_D where n is the theoretical number of ligand binding sites per receptor molecule, K_D the apparent dissociation constant, B_{max} the maximum number of binding sites as determined by Scatchard analysis, [L] the Molar concentration of radioligand, and B the amount of radioligand bound at a given concentration.

Values are represented as the mean $\pm$ standard error of the mean (SEM) and differences were assessed using the Students's t-test (two-tailed) when applicable, where a $P<0.05$ was taken to indicate a significant difference.

CHAPTER IV

EXPERIMENTAL RESULTS

Isolation and Purification of Mitochondrial Fractions

The investigation of [^{3}H]NBMPR binding to adenosine transport sites in mitochondria required a purified mitochondrial preparation. Since obtaining sufficiently pure mitochondrial fractions from heart tissue had proven unsuccessful in the past (Chance and Hagihara, 1961; Sordahl and Schwartz, 1967; Tyler and Gontz, 1967; Brierley et al., 1968; Matlib et al., 1983), a protocol was developed to obtain a fraction pure enough to be used in the binding assays. In developing the protocol, the isolation of mitochondria was carried out using various buffers: Tris-HCl (pH 7.0); Tris maleate (pH 7.4); potassium phosphate (pH 7.0); buffered sucrose (pH 7.2); mannitol (pH 7.4) and SMET (pH 7.4). Isolations using Tris-HCl and Tris maleate produced very small pellets with protein concentrations significantly less than 0.1 mg/ml, whereas buffered sucrose and mannitol buffers resulted in a higher yield. While the yield was higher, the mitochondrial pellet appeared broken and damaged as indicated by the light brown to beige color and very fluffy, soft texture. Furthermore, electron microscopic

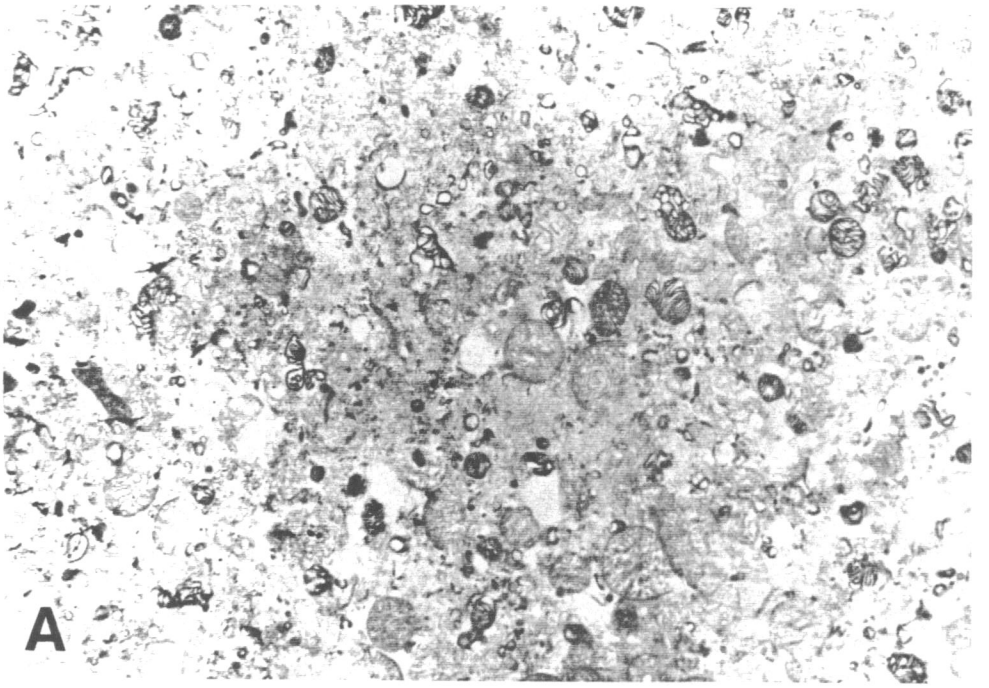
examination of mitochondria isolated in buffered sucrose revealed a large number of swollen mitochondria with ruptured or lysed outer membranes and artifactual remnants from other subcellular organelles (shown in Fig. 4). Additional artifacts that included blebbing and liposomal extrusions were also evident and are also seen in Fig. 4. These findings are consistent with the effect of sucrose and phosphate buffers on mitochondria as reported in the literature (Schnaitman and Greenwalt, 1986; Tzagoloff, 1982). In accord with reports in the literature, the SMET buffer produced mitochondrial pellets that exhibited a significant yield, higher protein concentrations, and good appearance (Nedergaard and Cannon, 1979; Tzagoloff, 1982; Dhalla et al., 1984).

Determination of Purity of Mitochondrial Fractions

The isolation procedure used to extract mitochondria from guinea pig ventricles yielded a substantially pure fraction, and electron microscopic examination of this fraction revealed intact mitochondria with minimal contamination by other subcellular organelles (see Figure 5). Further examination of the crude homogenate, pooled supernatants and the mitochondrial suspension via marker enzymes yielded additional evidence to support the finding of minimal contamination. The data in Table 3 indicate

Figure 4. TEM Micrographs of Mitochondria Isolated from Guinea Pig Ventricular Tissue.

The mitochondrial preparation shows artifactual remnants and contamination by other subcellular organelles. Some autolysis is evident (4-A). The isolation was done in a buffered sucrose solution (x10,200). Similar results occurred using a potassium phosphate buffer (4-B). Contamination has been reduced but autolysis is still prominent. Several cells are swollen (x10,200).


A

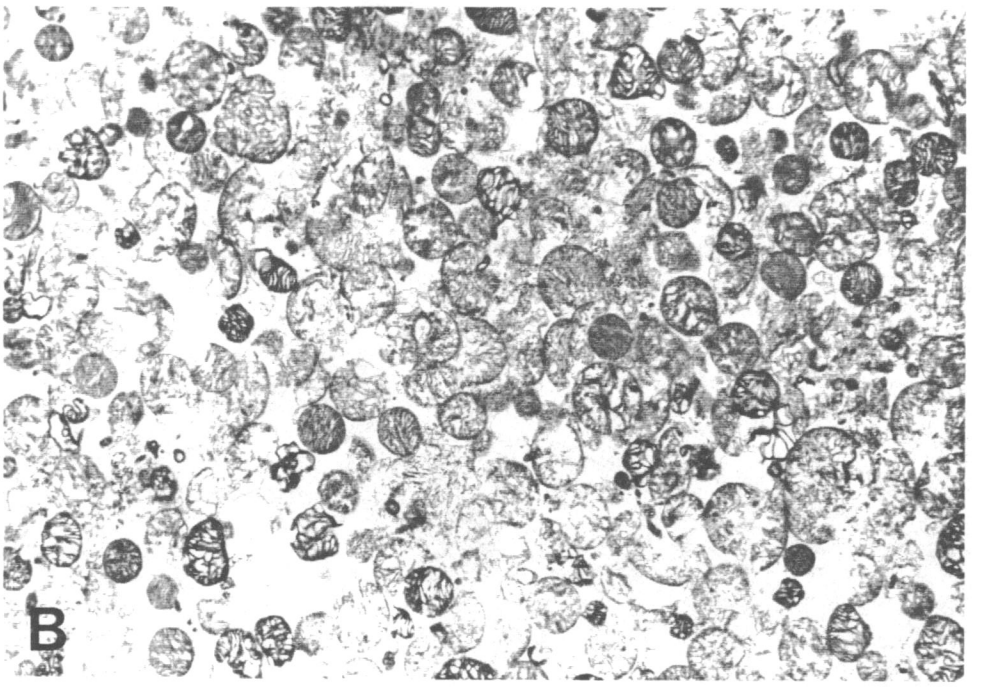

B

Figure 4. TEM Micrographs of Mitochondria Isolated from Guinea Pig Ventricular Tissue.

Evidence of swollen cells, autolysis, and contamination by sarcoplasmic reticulum can be seen in 4-C (x10,200).

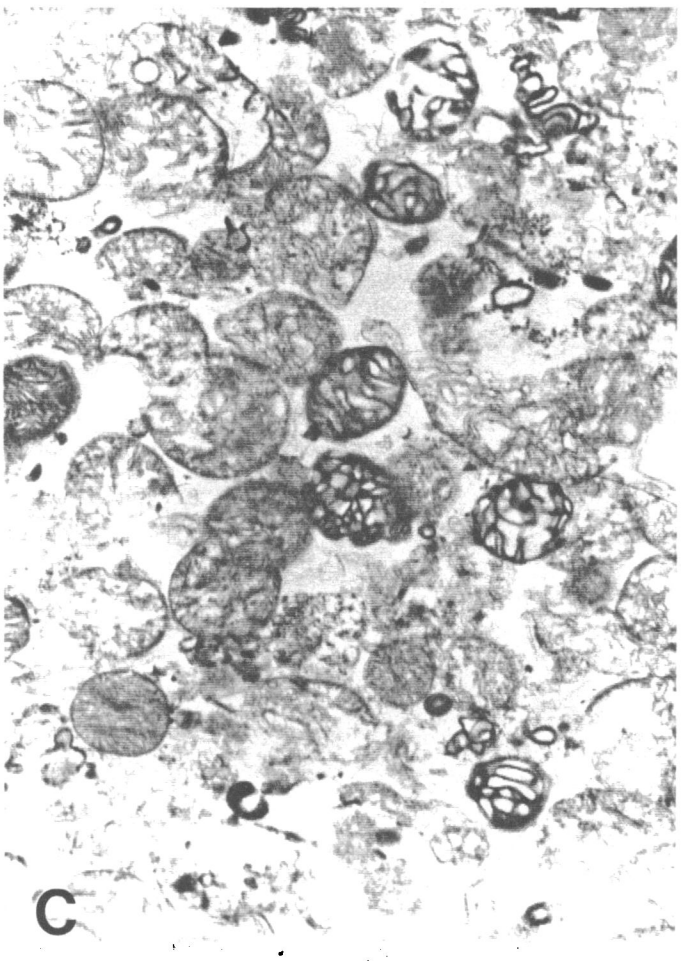

C

Figure 4. TEM Micrographs of Mitochondria Isolated from Guinea Pig Ventricular Tissue.

Micrograph 4-D shows a fairly clean preparation of intact mitochondria with evidence of blebbing and mitochondria undergoing proliferation. Autolysis and swelling have been reduced (x4,400).

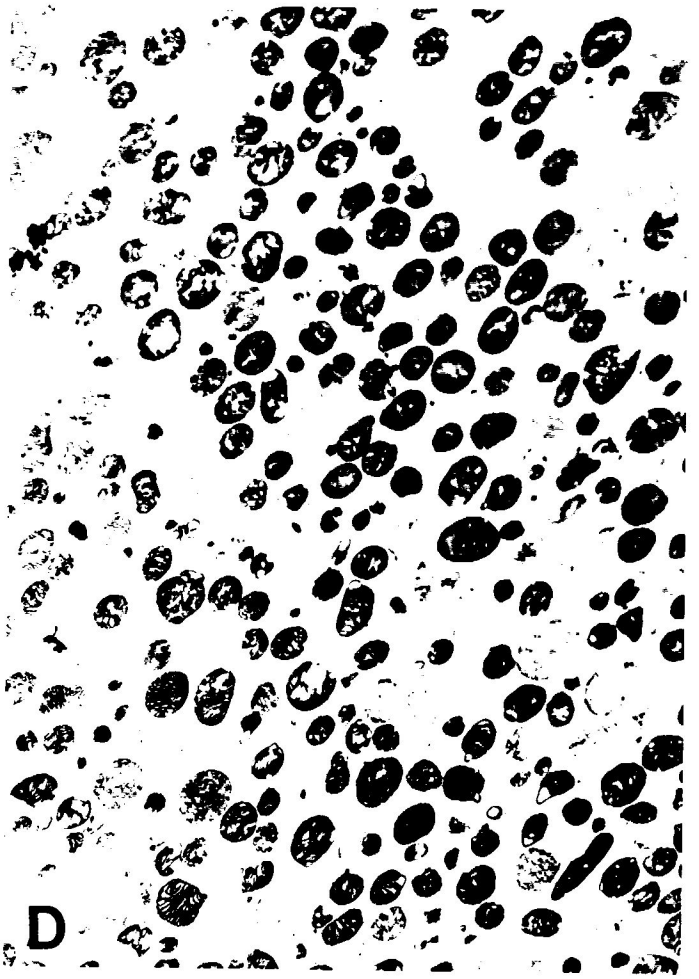
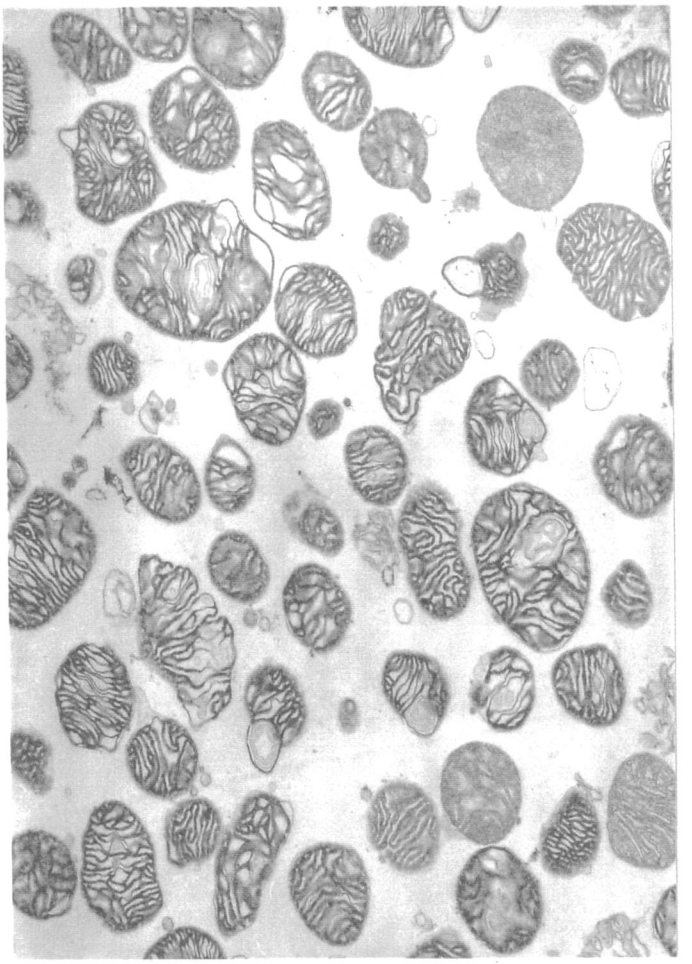

D

Figure 5. TEM Micrographs of Intact Cardiac Mitochondria Isolated from Guinea Pig Heart.

The figure shows a relatively pure fraction of intact mitochondria with many well developed cristae (x10K).



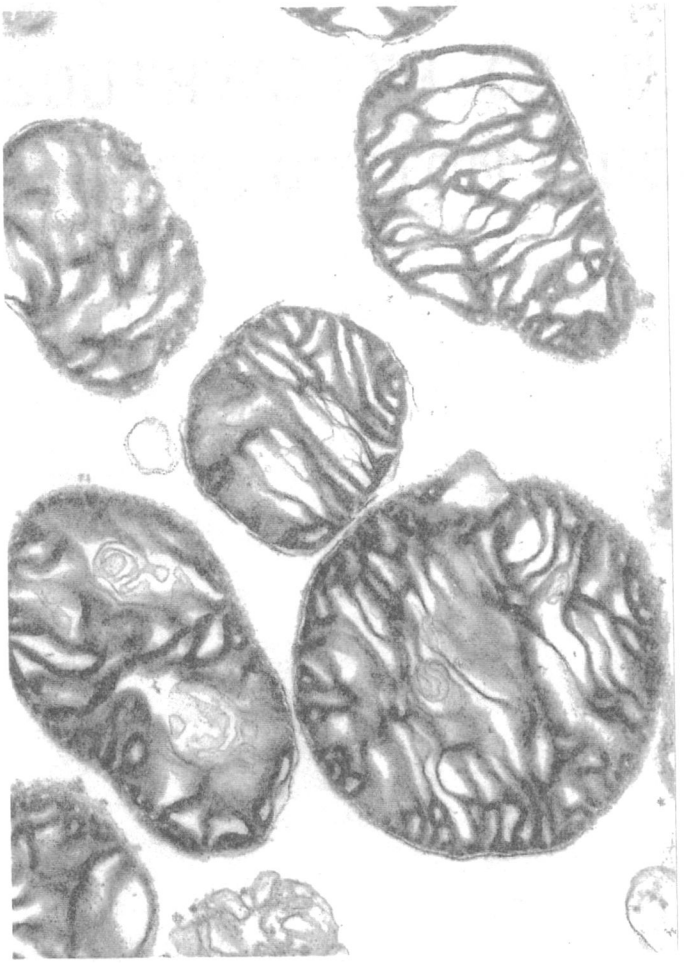


Table 3. Marker Enzyme Activities in Mitochondrial Fractions of Guinea Pig Heart

Enzyme Activity	Fraction CHM	PS	MT
Na^+-K^+-ATPase	11.1± 0.33	0.88± 0.02	0.33± 0.02
Ouabain-Sensitive-Na^+-K^+-ATPase	6.9± 0.44	3.8 ± 1.20	0.43± 0.02
Ca^{++}-Stimulated-Mg^{++}-ATPase	4.5± 0.08	0.58± 0.03	0.31± 0.07
Cytochromc C Oxidase	0.180±0.003	0.095±0.004	0.788±0.010

The enzyme activities were determined as described in Materials and Methods. All APTase activities were expressed as µmoles Pi/mg protein whereas the activity of cytochrome c oxidase was given as µmoles cytochrome c oxidase mg protein per min. Each value is a mean ± SE of 3 experiments using separate preparations. CHM= crude homogenate; PS = pooled supernatant; MT = mitochondria.

that the pooled supernatant and mitochondrial fractions contained very low Na^+-K^+-ATPase and Ca^{++}-Stimulated-Mg^{++}-ATPase activities with no appreciable differences between them as compared to the crude homogenate. There was however, a marked increase in Ouabain-sensitive-Na^+-K^+-ATPase activity in the pooled supernatant in comparison to the mitochondrial fraction. With regard to cytochrome c oxidase, specific activity in the mitochondrial fraction was enriched approximately 4-fold with respect to the corresponding enzyme activity in the crude homogenate (0.7782 ± 0.010 µmol/mg protein per min and 0.1801 ± 0.003 µmol/mg protein per min, respectively). These findings appear to confirm that the preparations employed in this study are enriched with relatively pure intact mitochondria (Dhalla et al., 1976; Palmer et al., 1977; Sordahl and Stewart, 1980).

Binding Parameters for Radioligand Equilibrium Binding Assay

With the exception of protein concentration, association and dissociation binding kinetics, all binding parameters used were identical to those employed in the established binding assay of Williams and colleages (1984) as described in Materials and Methods. Subsequent protein

linearity studies determined the optimal protein concentration to range from 0.20-0.40 mg protein per ml.

Characterization of [^{3}H]NBMPR Binding to Guinea Pig Cardiac Mitochondria

Kinetics of [^{3}H]NBMPR Binding to Mitochondrial Fractions

The kinetics of association and dissociation of [^{3}H]NBMPR binding to guinea pig cardiac mitochondria is illustrated in Figure 6. Binding reached equilibrium with 0.20 nM [^{3}H]NBMPR at 30 min of incubation at room temperature and remained at equilibrium for at least 120 min (Fig. 6). However, incubation was continued for 45 min to ensure the attainment of equilibrium and optimal binding levels. The initial rate constant (K_{obs}), calculated using the LIGAND computer program, was 0.439 min^{-1}. The addition of 30 uM unlabeled NBMPR completely reversed binding (Fig. 7) with a dissociation rate constant (K_{-1}) of 0.037 min^{-1} (K_{-1} = 0.693/half time of dissociation). A K_D value of 0.018 nM was calculated ($K_D = K_{-1}/K_{+1}$, where $K_{+1} = K_{obs}-K_{-1}$). Nonspecific binding was less than 19% in all experiments.

Analysis of the saturation data yielded nonlinear plots and a representative Scatchard plot of the nonlinear saturation isotherm for [^{3}H]NBMPR binding is shown in Figure 8. Computer analysis of the data revealed

Figure 6. Association Curve for [^{3}H]NBMPR Binding to Guinea Pig Cardiac Mitochondria.

Mitochondrial fractions were incubated with 0.20 nM ^{3}H]NBMPR at room temperature, filtered and then washed at the times indicated. Binding equilibrium was reached in approximately 30 min. K_{obs} was determined to be 0.43_{min-1}.

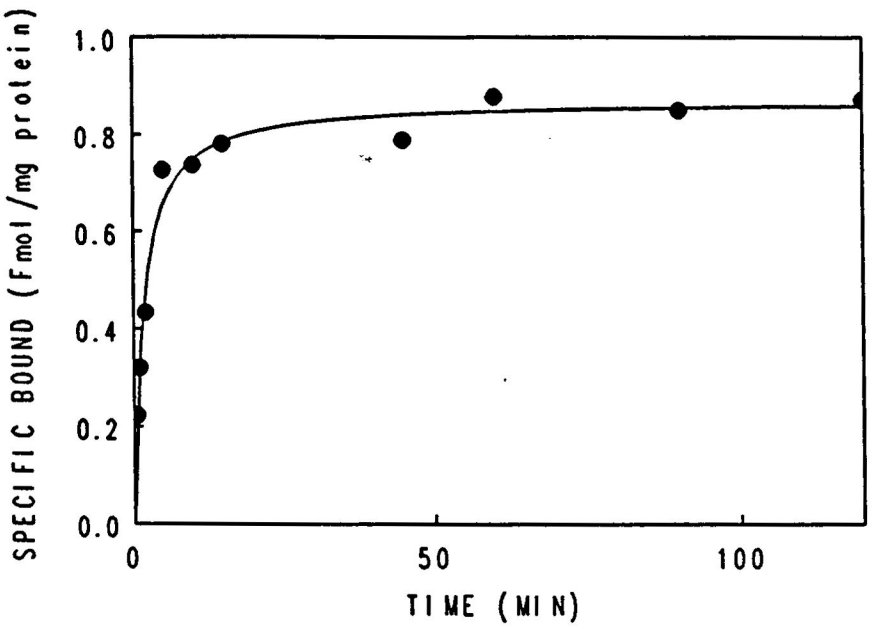

SPECIFIC BOUND (Fmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
TIME (MIN)

Figure 7. Dissociation Curve for [^{3}H]NBMPR Binding to Guinea Pig Mitochondria.

Unlabeled NBMPR (30 μM) was added to mitochondria that had been incubating for 60 min with [^{3}H]NBMPR. Binding was completely reversed with a $t_{\frac{1}{2}}$ of 18.7 min.

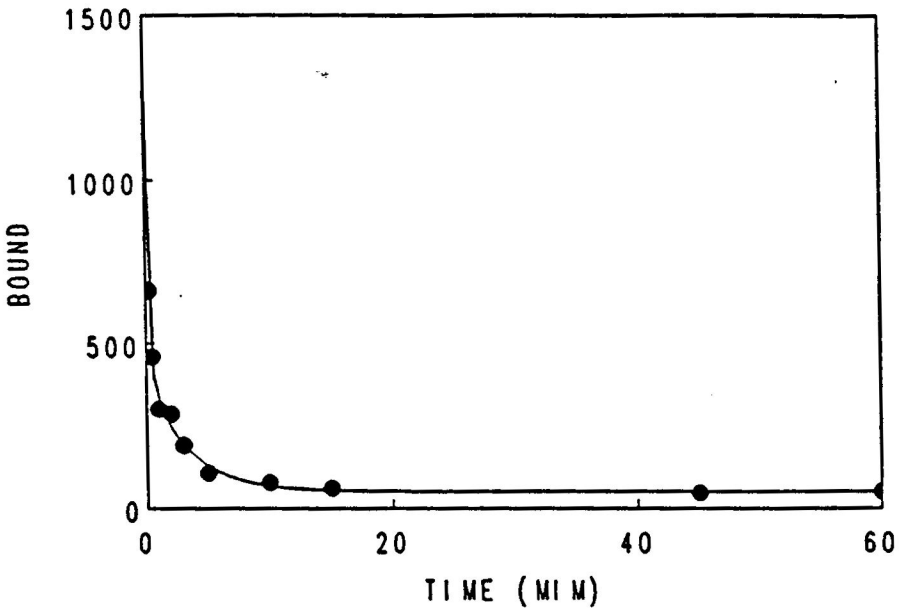
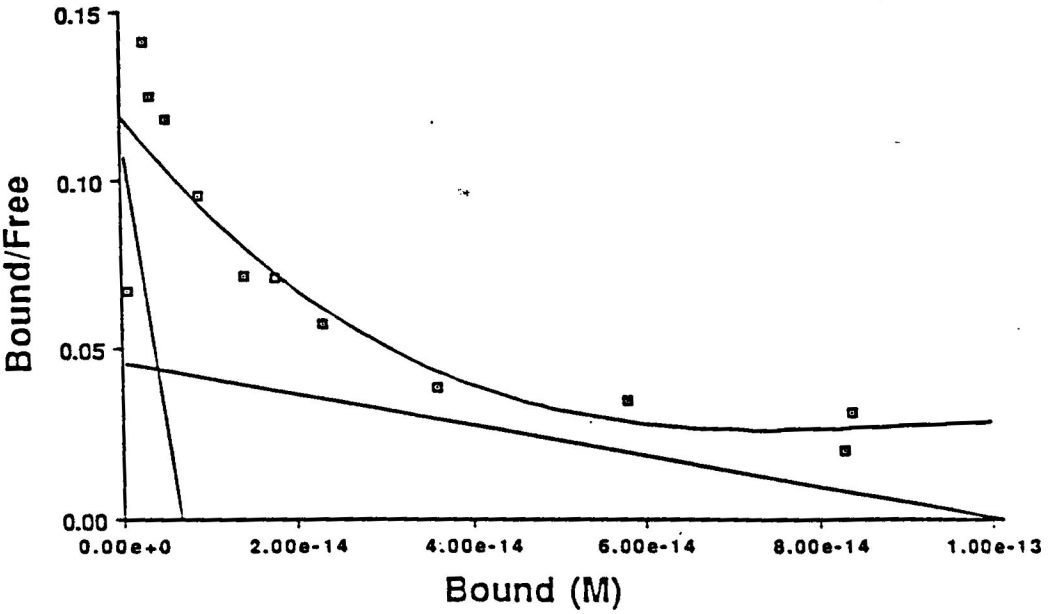

1500
1000
500
0
BOUND
0
20
40
60
TIME (MIM)

Figure 8. Representative Scatchard Plot of Saturation Isotherms of [^{3}H]NBMPR Binding to Guinea Pig Cardiac Mitochondrial Fractions.

Mitochondrial fractions were prepared and assayed as described in Materials and Methods. Scatchard plots were nonlinear which may indicate the presence of 2 classes of binding sites. Specific binding was measured as described under Materials and Methods. All assays were performed in duplicate, and this figure is representative of similar experiments conducted 4 times.


0.15
0.10
0.05
0.00
Bound/Free
0.00e+0
2.00e-14
4.00e-14
6.00e-14
8.00e-14
1.00e-13
Bound (M)

curvilinear plots which may suggest the presence of two classes of binding sites. These curvilinear Scatchard plots (n=4) were analyzed by the LIGAND computer program (Fig. 8) and found to be consistent with the presence of two classes of binding sites with dissociation constants (K_D) values of 0.006 nM $\pm$ 0.002 and 0.029 nM $\pm$ 0.012 and receptor density (B_{max}) values of 17 $\pm$ 2 fmol/mg protein and 177 $\pm$ 27 fmol/mg protein for the high and low affinity sites, respectively. The Hill coefficient values estimated from Hill plot analysis of this data ranged from 0.99-1.04, suggesting no cooperativity between binding sites (Figure 9). This observation may be indicative of sites that interconvert between high and low affinity states for the ligand (Klotz et al., 1989; Williams et al., 1990).

Because binding appeared biphasic with intact mitochondrial fractions, studies using the crude homogenate and pooled supernatants were done to determine if binding was similar. All Scatchards studied appeared biphasic and the data plus Hill coefficients are found in Table 4. The highest density of binding sites was located in the homogenate fraction and the lowest in the mitochondrial fraction.

Additional experiments were performed in an attempt to clear up specific concerns and discrespancies about the

Figure 9. Hill Plot of [^{3}H]NBMPR Binding on Cardiac Mitochondria.

Hill plot analysis of the saturation data resulted in Hill coefficients of the Hill slopes ranging from 0.99-1.04. The plot is representative of 4 experiments that yielded similar results. Hill coefficients for the saturation data are also found in Table 4.

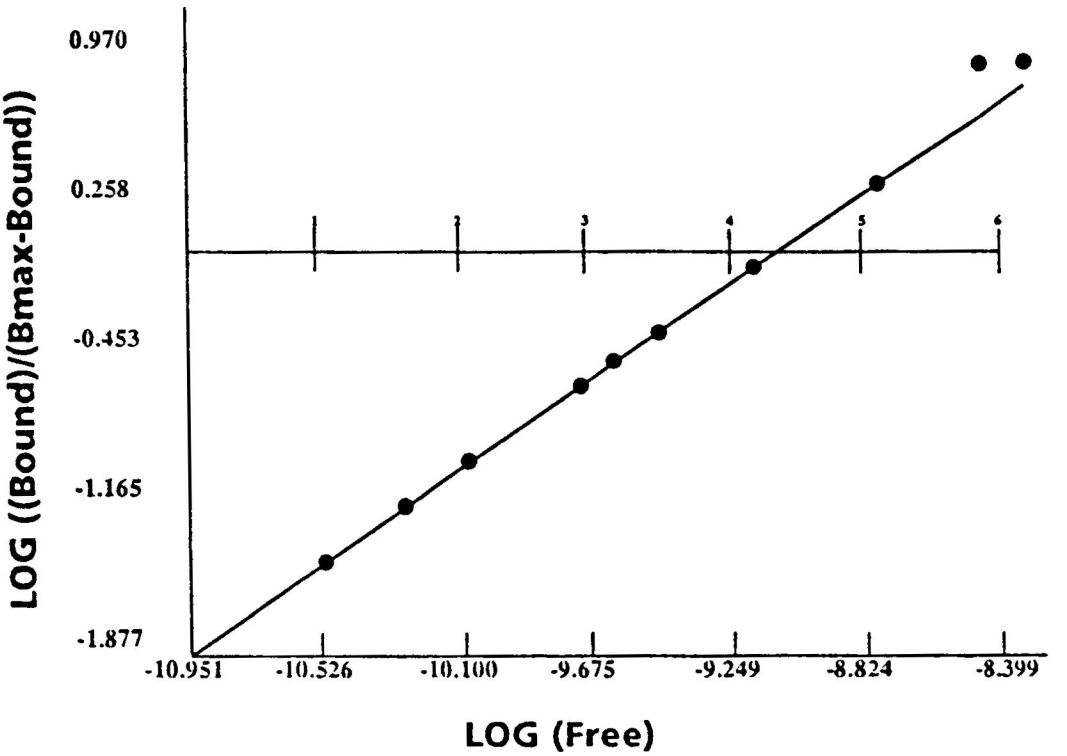

0.970
0.258
-0.453
-1.165
-1.877
LOG ((Bound)/(Bmax-Bound))
-10.951
-10.526
-10.100
-9.675
-9.249
-8.824
-8.399
LOG (Free)

Table 4. [^{3}H]NBMPR Binding Parameters in Fractions of Guinea Pig Ventricular Tissue

	Specific Binding		
Fraction	B_{max} (fmol/mg protein)	K_D (nM)	n_H
MT (High Affinity Site)	17± 2.000	0.006±0.002	1.04
(Low Affinity Site)	177±27.000	0.029±0.012	0.99
PS (High Affinity Site)	29± 9.000	0.002±0.001	1.01
(Low Affinity Site)	188±13.000	0.006±0.002	0.98
HM (High Affinity Site)	15± 3.000	0.003±0.001	0.97
(Low Affinity Site)	229±38.000	0.008±0.006	0.93

Fractions were prepared as described in Materials and Methods. Binding parameters were determined by Scatchard analysis using LIGAND computer program. The experiment was repeated 4 times with similar results. The data presented are means ± S.E. of the means. n_H= Hill coefficient. HM = Homogenate; PS = Pooled Supernatant; MT = Mitochondria. There was no statistical difference between the fraction ($P>0.05$).

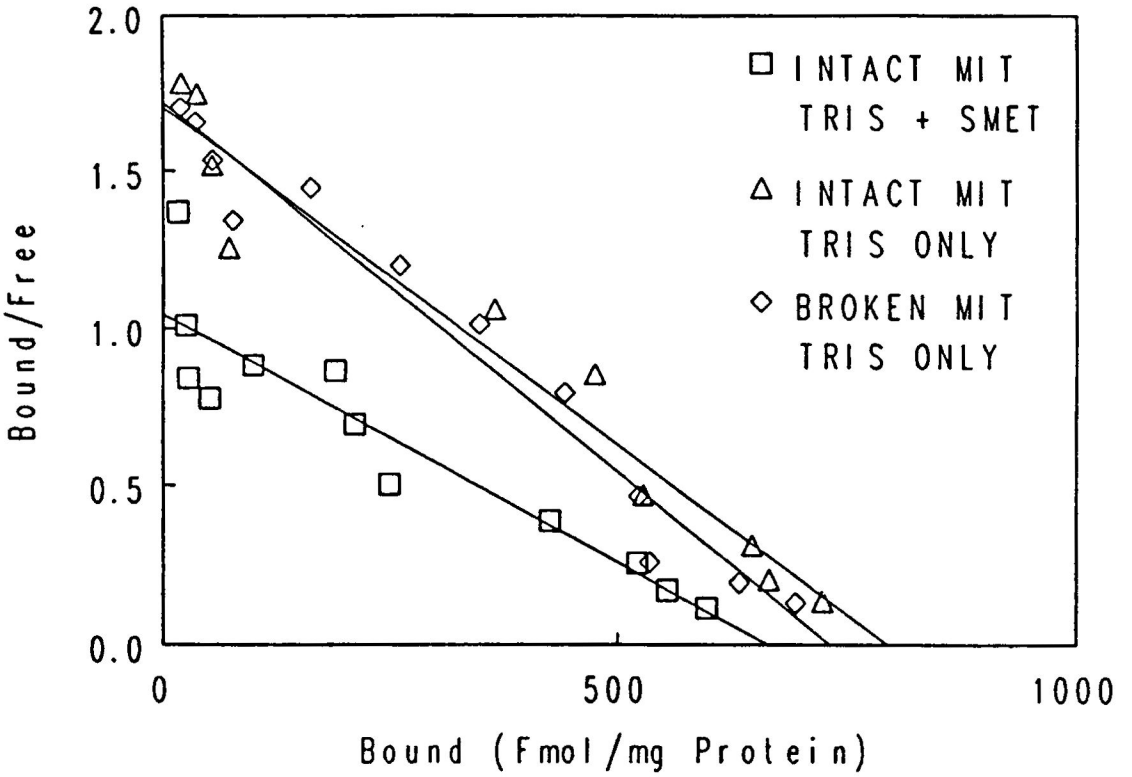
use of SMET buffer in the radioligand binding assays. The radioligand binding assays were repeated under varied conditions. One experiment involved binding of [^{3}H]NBMPR on mitochondrial pellets that had been washed in SMET and then suspended in Tris-HCl. The homogenization procedure was not altered. Another variation involved performing binding experiments on intact mitochondria that had been washed and suspended in Tris-HCl and homogenized as in the original preparations. A third experiment was done exactly as above except that the pellet was homogenized at an increased speed with the Polytron homogenizer. The gentle disruption with the Potter-Elvejhem homogenizer was omitted in order to produce broken mitochondrial membranes. Scatchard analyses of the data revealed linear Scatchard plots in all preparations studied. Concurrently, a similar and significant increase in binding site densities was observed in all preparations (B_{maxes} of 589 fmol/mg protein, 793 fmol/mg protein, and 731 fmol/mg protein in experiments 1, 2, and 3, respectively) as compared to the nonlinear Scatchards of the original preparations (B_{max}=194 fmol/mg protein). The results of these experiments are shown in Figure 10.

Selectivity of [^{3}H]NBMPR Binding Sites

Displacement and pharmacology studies for various adenosine analogs, xanthine antagonists and nucleotides

Figure 10. **Scatchard Analysis of [^{3}H]NBMPR Binding to Broken and Intact Mitochondria using Tris-HCl Buffer.**

Increasing concentrations of [^{3}H]NBMPR were incubated with broken and intact mitochondria prepared as described in the text. Data points represent the results from single experiments. See text for the B_{max} and K_D values.


2.0
1.5
1.0
0.5
0.0
0
500
1000
Bound/Free
Bound (Fmol/mg Protein)
INTACT MIT
TRIS + SMET
INTACT MIT
TRIS ONLY
BROKEN MIT
TRIS ONLY

tested are illustrated in Table 5 and the IC_{50} values are listed in Figure 11. The adenosine transport inhibitors were the most potent inhibitors of [^{3}H]NBMPR binding. NBMPR and NBTGR were equipotent (IC_{50}=0.001 μM) and were 44 times more potent than dipyridamole (I_{C50}=0.044 μM). Adenosine (IC_{50}=1.29 μM) was approximately twice as potent than the most potent A_1 receptor ligand CPA (IC_{50}=2.28 μM) which was 11 times more potent than CHA, the agonist next in potency (IC_{50}=25 μM)in inhibiting binding. In addition, S-PIA was twice as potent as its stereoisomer R-PIA which is not in agreement with a previous report on the selectivity of these two compounds (Stone, 1985). The agonist rank order of potency for the A_1-receptor ligands was: CPA>CHA>S-PIA>R-PIA. In comparison, MECA was the most potent A_2 receptor ligand (IC_{50}=1.74 μM). The agonist rank order of potency for the A_2-receptor ligands was: MECA>CV-1808>2CADO>CPCA. While the adenosine receptor ligands were approximately 5 orders of magnitude less potent in inhibiting binding, a fairly extensive list of purines, nucleotides and methylxanthines were poor inhibitors of NBMPR binding at the concentrations tested (see legend in Table 5). IC_{50} values for NECA, papaverine, caffeine and theobromine could not be calculated because their inhibition profiles were not clearly dose responsive.

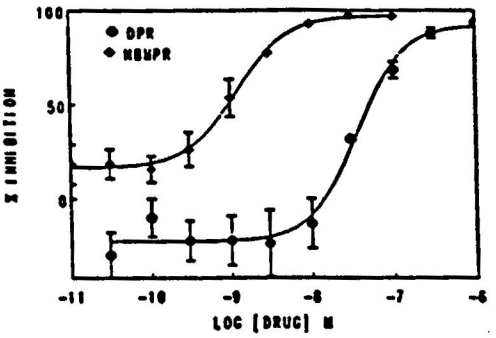
Table 5. Pharmacology of [^{3}H]NBMPR Binding in Guinea Pig Cardiac Mitochondria

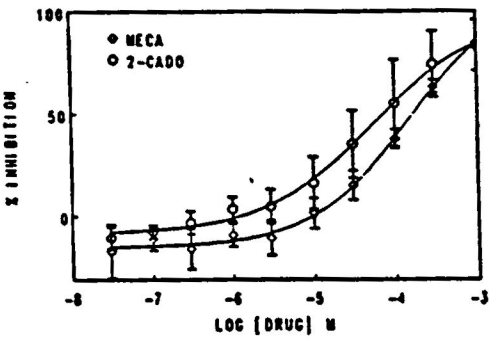
Inhibitor	IC_{50} (μm)	n_H
A_1 Receptor Ligands		
N^6-Cyclopentyladenosine (CPA)	2.28 ± 0.09	0.645
N^6-Cyclohexyladenosine (CHA)	25 ± 0.05	1.020
R-Phenylisopropyladenosine (R-PIA)	154 ±35.00	0.596
S-Phenylisopropyladenosine (S-PIA)	94 ±28.00	0.764
A_2 Receptor Ligands		
2-Phenylaminoadenosine (CV-1808)	1.87 ± 0.18	1.180
2-Chloroadenosine (2-CADO)	24 ± 1.90	0.612
5'-N-Methylcarboxyamidoadenosine (MECA)	1.74 ± 0.09	0.669
5'-N-Ethylcarboxyamidoadenosine (NECA)	nc	
Cyclopropylcarboxyamidoadenosine (CPCA)	136 ± 0.05	0.693
Adenosine Receptor Antagonists		
Dimethylxanthine (Theobromine)	nc	
Caffeine	nc	
Adenosine Transport Inhibitors		
Nitrobenzylthioinosine (NBMPR)	.001 ± 0.0002	1.250
Nitrobenzylthioguanosine (NBTGR)	.001 ± 0.0008	0.974
Dipyridamole (DPR)	.044 ± 0.0030	2.280
Nucleosides		
Adenosine	1.29 ± 0.057	0.801

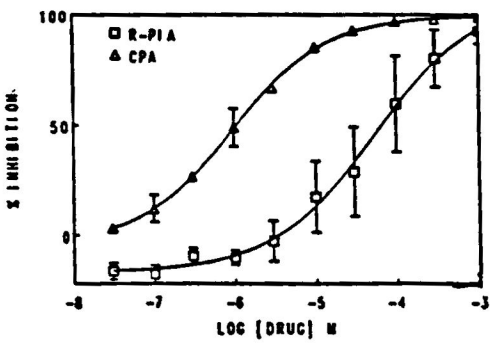
The IC_{50} value for each inhibitor was determined by testing 10 concentrations for inhibition of [^{3}H]NBMPR binding (0.20 nM). Each compound was tested at least twice in duplicate and the values were averaged. GraphPAD InPlot computer program was used to calculate IC_{50} values and Hill cofficients (n_H). A number of compounds were tested that showed no appreciable effect on binding at up to 1 mM. These included: nucleotides - ADP, AMP, GMP, GDP, ATP, cAMP, and cGMP. Inhibition of NECA, Theobromine, and Caffeine did not appear to be dose responsive. nc = not calculated.

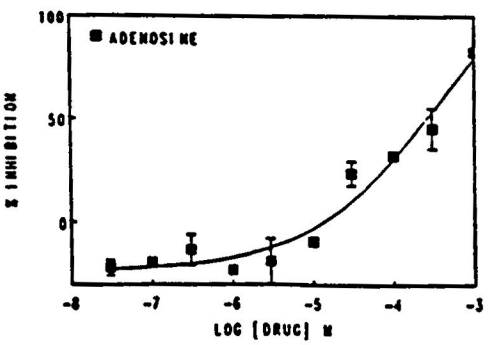
Figure 11. Displacement Curves of [^{3}H]NBMPR Binding by Nucleoside Transport Inhibitors and Adenosine Analogs.

IC_{50} values for various adenosine agonists and antagonists, nucleoside transport inhibitors, and nucleotides were determined using 0.20 nM [^{3}H]NBMPR and 10 concentrations of the test compounds. These results represent 3 experiments with similar results. IC_{50} values were calculated using GraphPad InPlot computer program. Shallow curves are generally indicative of Hill coefficients <1 which suggest the presence of more than one class of binding sites.


100
50
0
% INHIBITION
DPR
NBMPR
-11
-10
-9
-8
-7
-6
LOG [DRUG] M


100
50
0
% INHIBITION
MECA
2-CADO
-8
-7
-6
-5
-4
-3
LOG [DRUG] M


100
50
0
% INHIBITION
R-PIA
CPA
-8
-7
-6
-5
-4
-3
LOG [DRUG] M


100
50
0
% INHIBITION
ADENOSINE
-8
-7
-6
-5
-4
-3
LOG [DRUG] M

CHAPTER V

DISCUSSION

Nucleoside transport is a facilitated diffusion system which can transport a variety of nucleosides. Several studies have been done in an attempt to fully characterize this somewhat simple carrier-mediated transport system in order to better understand the role of nucleoside transport and adenosine in cardiovascular function. However, problems have persisted in the past (i.e., the high turnover rate of the nucleoside transporter, and the limited existence of radiolabeled inhibitors of nucleoside transport that can be used to characterize nucleoside transport systems and the rapid uptake of nucleosides) that contributed to the difficulty in measuring transport. Thus, it is not surprising that the available data regarding nucleoside transport in different systems have been subject to different interpretations that resulted in uncertainties about the role of nucleoside transport systems.

Nucleoside transporter function employing radioligand probes and radioligand binding have been investigated in a variety of crude membranes, intact cells, and cellular

fractions. Although the studies have been informative, there is still much to learn about nucleoside transport and its role in different physiological systems.

To further investigate the role of adenosine and nucleoside transport in cardiovascular function, the characterization of adenosine transport sites in guinea pig cardiac mitochondria using [^{3}H]NBMPR as a radioligand probe was studied. Interestingly, this is the first attempt (to this investigator's knowledge) to examine nucleoside transport in mitochondria even though the organelle may probably participate in more metabolic functions than any other cellular organelle. As previously stated, a requirement for binding studies at the subcellular level is a relatively pure subcellular fraction. The fulfillment of this requirement has been difficult to accomplish in the past. The tough collagenous fibers that make up cardiac tissue made tissue disruption difficult to accomplish without decreasing the amount of tissue yield and recovery of mitochondrial protein. Thus a major effort and considerable time were spent on developing a protocol that would yield a substantially pure mitochondrial preparation.

Various methods described for the isolation of heart mitochondria from the rat (Cleland and Slater,

1953; Montgomery and Webb, 1956), rabbit (Harman and Feigelson, 1952; pigeon (Chance and Hagihara, 1961) and dog (Jaqua-Stewart et al., 1978) were studied. The methods of Chance and Hagihara employed brief proteinase (B. subtilis) digestion of tissue slices followed by mechanical homogenization. This technique produced reasonably good yields of functional mitochondria devoid of other contaminating organelles and became the method of choice for the preparation of heart mitochondria from laboratory animals. Tyler and Gonze (1967) followed the lead of Chance and Hagihara and employed proteinase (Nagarse enzyme) in their preparatory procedure. The use of proteinase subsequently became controversial when it became evident that Nagarse was not highly selective in its degradative actions and yielded mitochondria that displayed protease damage. Another problem that developed with the use of Nagarse involved the prolonged incubation periods of the tissue (excess of 5 min) and the necessity of relatively high concentrations of the enzyme, which often resulted in mitochondrial damage. Additionally, temperature had to be strictly regulated in order to maintain the functional capabilities and integrity of the mitochondria. As a result, alternate methods were then designed that discontinued the used of Nagarse (Matlib et

al., 1978). However, a satisfactory isolation procedure was still unavailable.

Isolation procedures were proposed to significantly enhance or decrease tissue yield and tap different population of mitochondria from tissue (Palmer et al., 1977; Shimada et al., 1984). Subsequently, different populations of mitochondria were reported in ventricular myocardium of rats and Japanese monkeys. In this regard, Palmer and colleages combined isolation techniques and isolated two populations of mitochondria, one clustered beneath the sarcolemma (subsarcolemmal) and another located between the myofibrils (interfibrillar). Subsarcolemmal mitochondria were isolated via treatment of heart muscle mitochondria with a Polytron homogenizer, while interfibrillar mitochondria were released by Nagarse digestion of the remaining tissue. Each type of mitochondria differed not only in their location but in their biochemical properties as well. Monkey myocardium was reported to contain a third population of mitochondria located around one pole of the nucleus (perinuclear) in addition to the subsarcolemmal and interfibrillar mitochondria (Shimada, 1984).

Since the main obstacle in isolating pure heart mitochondria was the fibrous make up of cardiac tissue,

methods of other investigators (Chance and Hagihara, 1961 as modified by Tyler and Gonze, 1967; Chance and Hagihara, 1961; Eastabrook and Pulman, 1967; Jacqua-Stewart et al., 1978; Mela and Seitz, 1979; Sordahl and Stewart, 1980) were attempted without success by this author. A consistent problem encountered was in the method of tissue disruption and choice of buffers. In earlier isolation procedures, Nagarse digestion of the tissue in all buffers used resulted in extremely small pellets that appeared to have layers of damaged mitochondria or some other material suspended on top of each other. Several buffers (Tris maleate, buffered sucrose, dilute potassium phosphate, Tris-HCl, KEA (KCl, EGTA and BSA), and SMET were employed in the isolation procedures. With the exception of the SMET buffer, pellets were produced that were low in mitochondrial protein concentrations, of poor quality in color, size, and texture, and that often adhered to the sides of the centrifugation tube. The tubes had to be shaken in order to loosen the pellets, which resulted in their breakage. Another problem involved tissue disruption. Homogenization problems occurred when the tissue was disrupted in the absence or presence of Nagarse. These problems included incorrect speed of homogenization that prevented the production of

an even homogenate without mitochondrial damage; excessive polytron homogenization; improper dilution of tissue before and after homogenization with the Potter-Elvejhem homogenizer; and undue delay in carrying out the entire isolation procedure. A final problem to be resolved was that of centrifugation speed. Earlier centrifugations were too fast, which caused the mitochondria to sediment out with lipids and other contaminants. Some of the mitochondria may have also sedimented out in nuclear fractions which were discarded and could explain the low tissue yield, in some instances.

After numerous attempts and many trials and errors, a protocol (Fig. 3) was developed that was a modification of the procedures of Jaqua-Stewart et al. (1978) and Chance and Hagihara (1963) as modified by Tyler and Gonze (1967), which yielded a mitochondrial fraction from guinea pig heart tissue that was about 4-5 times purer than the crude homogenate as verified by enzyme marker assays (Table 3). This finding is similar to those suggested by other investigators (Fleischer et al., 1978; Dhalla, 1983). Moreover, electron microscopic examination revealed a heterogeneous fraction that appeared to consist of perinuclear mitochondria and some subsarcolemmal mitochondria. These mitochondrial populations were

identified based on the morphological description reported by Shimada and colleages (1984).

Surprisingly, a novel finding of this study was the demonstration of [^{3}H]NBMPR binding in mitochondrial fractions, even though Scatchard analysis of the saturation data were nonlinear, suggesting two classes of binding sites. These nonlinear or curvilinear Scatchard plots may have been induced by SMET buffer as a result of the sucrose and/or mannitol interfering with binding, thereby, lowering the binding site density. However, it is difficult to accept this hypothesis without further investigations because it is logical to postulate, from a structural-activity perspective, that the NBMPR is binding to some other site rather than to nucleoside transport sites. An alternative suggestion is that NBMPR may only be able to bind to a small number of transporter sites in intact mitochondria or that the organelle may only possess a very small number of binding sites at the onset. Disrupting the mitochondria, allows the probes access to internal binding sites that override the surface binding. Another avenue to consider is the incubation procedure in the assay. Since the incubation of the mitochondria was carried out at room temperature, allowing for subsequent internalization of the probe, the rapid uptake of the probe would then allow it to label internal high and low

affinity sites. Furthermore, performing the assay at room temperature with intact mitochondria could also lead to further metabolism of the radioligand which was analyzed in addition to transport.

The additional experiments using Tris-HCl buffer instead of SMET yielded expected results. When intact mitochondria were exposed to Tris, their ability to remain intact was eliminated. Tris, which is hypotonic to mitochondria, induced swelling and lysis of the intact organelles. Once this occurred, NBMPR was able to bind to a conglomerate of heterogenous material and membranes which resulted in the production of linear Scatchard plots with increased binding sites. However, the actual binding constituents were not determined.

To further explore the possibility that sucrose, mannitol, or SMET buffer interfered with binding, an additional experiment was performed using broken membranes. The data revealed that binding was decreased (up to 75%) when sucrose, mannitol, or SMET was added to the assay instead of Tris-HCl. A reasonable explanation to consider is that the mitochondria had not been subjected to a Polytron rheostat setting high enough or long enough to ensure that the membranes were completely broken. Consequently, the addition of SMET, sucrose, or mannitol to these broken mitochondria caused the membranes

to reseal, thereby causing a decrease in binding when compared to Tris.

Experiments characterizing the binding of [^{3}H]NBMPR to guinea pig heart mitochondria demonstrated that binding was saturable with time and reversible. The dissociation curve was nonlinear with a $t_{1/2}$ of 18.7 min indicating the possible existence of more than one dissociation site. Similarly, the nonlinear Scatchard plots of the binding isotherm data suggest the possibility of two classes of binding sites with high and low affinity for the ligand. A K_D value of 0.018 nM was calculated from the association and dissociation rate constants which is in close approximation of the K_D value of 0.029 nM of the low affinity site determined by Scatchard analysis of the saturation data. This observation may be due to the presence of a higher number of binding sites in the lower affinity state that constituted a large proportion of the total binding activity. Thus, the binding kinetics of these low affinity states were more accurately determined under these conditions.

Hill coefficient values (^nH between 0.99-1.04) suggest, however, that no cooperativity occurs between the binding sites which is generally indicative of a homogenous population of binding sites. One possible explanation for the discrepancies in the data, may be the

possible existence of binding sites that convert between high-affinity and low-affinity states. Additionally, these interconvertible states of binding sites have been reported in spare receptors of isolated rat fat cells using the agonist photoaffinity ligand R-APHIA (Lohse et al., 1986), in human umbilical vein endothelieum using [^{3}]NBMPR (Williams et al., 1990) and are also characteristic of agonist binding to beta-adrenergic receptors (Maquire et al., 1975; Lefkowitz et al., 1976; Kent et al., 1980).

Alternatively, the presence of two classes of binding sites may represent ligand binding to two different recognition binding sites on a single substrate macromolecule, or to closely related allosteric sites in cardiac mitochondrial preparations or possibly to more than one type of nucleoside transporter located in the preparations used. Moreover, the isolation procedure may also have produced some mitochondrial artifact that was sensitive to the radioligand probe which may support the hypothesis that different populations of mitochondria released from cardiac tissue based on the homogenization procedure, possess morphological and functional differences (Palmer et al., 1977). It is then conceivable, that binding might be somehow linked to the morphology of the mitochondrial population.

The degree of interaction of various adenosine receptor agonists and antagonists, adenosine transport inhibitors and nucleotides with [^{3}H]NBMPR was also examined. The rank order of potency of the adenosine receptor agonists (CPA>CHA>S-PIA>R-PIA= A_1 receptor and MECA>CV-1808>2CADO>CPCA= A_2 receptor) for the [^{3}H]NBMPR binding, fits neither the A_1 receptor profile (CPCA>CPA>CHA>R-PIA>2-CADO>NECA>S-PIA>CV-1808) or the A_2 receptor profile (NECA>MECA>2-CADO>CV-1808=R-PIA>CPA=CHA>S-PIA) (Varney and Skidmore, 1985; Burnstock and Kennedy, 1985; Bruns et al., 1987; Gilfillan et al., 1989; Trivedi et al., 1990). The interaction of [^{3}H]NBMPR with the A_1 receptor is also unlikely since S-PIA appeared to be twice more potent than its stereoisomer R-PIA, in displacing the radioligand which may indicate little, if any, stereoselectivity. R-PIA had been shown to be approximately 30-200 times more potent than S-PIA at the A_1 receptor in the brain and spinal cord membrane preparations (Daly, 1982; Geiger et al., 1984). Furthermore, the adenosine receptor antagonists and the nucleotides were all virtually ineffective on [^{3}H]NBMPR binding at concentrations up to 1 mM. This observation is interesting because ATP, ADP, 5'-AMP and theophylline have been shown to be inactive at the intracellular P site in brain and spinal cord (Daly, 1982). In comparison, all

IC_{50} values of the compounds studied, with the exception of the adenosine transport inhibitors (NBMPR, NBTGR, and Dipyridamole), adenosine, and the receptor ligands CV-1808 (A_2) and CPA (A_1), indicated low affinity of these compounds for the ligand. Interestingly, like the potent nucleoside transport inhibitors NBMPR and NBTGR, CHA and CV-1808 displayed Hill coefficients of 1 indicating that no cooperativity occurred between the sites. Taken together with the observations of Hill coefficients less than one in almost all the compounds, these findings can be interpreted on the basis of structure-activity relationships, tissue uptake and subsequent intracellular metabolism (Davies, 1982). The potent inhibitory actions of NBMPR and related compounds have been attributed to the 6-N-substituted nitrobenzyl and 9 ribosyl moities extending from the purine ring (Paterson and Oliver, (1971). Unlike the adenosine receptors, the P site cannot accept alterations in its purine ring. CHA and the stereoisomers R-PIA and S-PIA are similar to NBMPR in that they all possess the 9 ribosyl group as well as the hydrophobic ring structure at the N^6 position. Consequently, 2-CADO was less potent in displacing [^{3}H]NBMPR, possibly because it lacks the N^6 group. Thereby, when considering the biological actions of these receptor agonists, the inhibition of endogenous adenosine

uptake should be considered as a contributing factor to these effects.

Finally, a comparison study of [^{3}H]NBMPR binding parameters in guinea pig cardiac mitochondria and in crude guinea pig ventricular membranes was performed. Unlike cardiac mitochondria, [^{3}H]NBMPR bound to a high number of a single class of sites with high affinity (K_D =0.41 $\pm$0.08 nM and B_{max} =1802 $\pm$122 fmol/mg protein). Binding was rapid, reversible and saturable. The binding parameters of the crude ventricular membranes are in good agreement with values obtained from earlier studies in guinea pig cardiac membranes (Williams and Clanachan, 1983); guinea pig cortical synaptosomes (Hammond and Clanachan, 1982) and in human erythrocytes (Hammond et al., 1981).

A major finding of this investigation is that nucleoside transport sites can be labeled with [^{3}H]NBMPR in guinea pig cardiac mitochondrial membranes. This observation is a step towards augmenting our knowledge about the precise localization of cardiac nucleoside transporters as well as establishing a possible subcellular basis for the nucleoside transport mechanism in myocardial function. In accordance, further studies must be done and extended to the sarcolemma and sarcoplasmic reticulum in order to carry out a

comprehensive investigation of nucleoside transport at the subcellular level in cardiac tissue.

Interpretation of the [^{3}H]NBMPR binding data in guinea pig cardiac mitochondria is difficult since there have been no previous binding studies done and no physiologic evidence is available to indicate the presence of an adenosine transport site in this fraction. It does appear however, that some binding may be intracellular.

It is evident from the data generated and the questions raised as a result of this investigation, that the molecular and subcellular basis for adenosine's role in cardiovascular function and in nucleoside transport in the heart is still unanswered and that further study is warranted. However, the data generated suggest that the mitochondria may be employed as a potential model in which to study nucleoside transport in the heart even though the study should be extended to other subcellular fractions as well.

CHAPTER VI

SUMMARY AND CONCLUSIONS

In summary, this investigation involved the subcellular characterization of nucleoside transport binding sites in guinea pig cardiac mitochondria. The data yielded some interesting results and the following conclusions can be stated:

1) A specific transport process exists for nucleoside (e. g., adenosine) uptake by cardiac tissue via facilitated diffusion and is characterized by Michaelis-Menten enzyme kinetics.
2) The results indicate that intact mitochondrial fractions contain NBMPR binding activity mainly of low affinity.
3) The nucleoside transport inhibitor [^{3}H]NBMPR, binds specifically, saturably, and reversibly, to a transporter site that may be capable of interconverting between high and low affinity states that are selective for adenosine.
4) The displacement studies suggest that the sites labeled by [^{3}H]NBMPR do not correspond to any known adenosine receptor (i.e., A_1 or A_2) or to

the P site based on their pharmacological profiles and order of potencies.

5) Finally, the data demonstrate that [^{3}H]NBMPR binding occurs in guinea pig cardiac mitochondria even though the transport mechanism is unknown. However, it has been suggested that sufficiently high intracellular levels of adenosine may cause it to flow down its concentration gradient and pass out of cells through a nucleoside transport system (Belloni et al., 1985; Bukoski et al., 1986).

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